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**(54) Title:** MONOCLONAL ANTIBODIES NEUTRALIZING HIV-1

**(57) Abstract**

Monoclonal antibodies are disclosed which bind to the gp 120 protein on the envelope of HIV-1. These antibodies neutralize HIV-1. They inhibit the rate of infection of T cells, and also inhibit syncytium formation. Further, the antibodies are group-specific and neutralize different strains and isolates of HIV-1. These antibodies have a variety of uses, including the treatment and prevention of AIDS and ARC. Chimeric viral-neutralizing antibodies, including chimeric HIV-neutralizing antibodies, are also disclosed.

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MONOCLONAL ANTIBODIES NEUTRALIZING HIV-1Background of the Invention

Acquired Immune Deficiency Syndrome, generally known by its acronym AIDS, is probably the most serious health threat confronting society. The disease runs a painful and debilitating course and usually results in the death of its victim. In fact, from diagnosis onward, the average life span of an AIDS victim is less than two years,

To date, about 40,000 AIDS cases have been reported in the United States. Approximately two-thirds of individuals have died from the disease.

AIDS is caused by a virus which has at various times been called human T-cell lymphotropic virus type III (HTLV III), or lymphadenopathy-associated virus (LAV). The virus is currently known as human immunodeficiency virus I (HIV-1). It is estimated by the Center for Disease Control, U.S. Public Health Services, and the National Academy of Sciences that in the United States alone, about 1.5 million people will have been infected by 1991. The results from many long-term epidemiological studies indicate that twenty to sixty percent of the infected group will develop AIDS within the next five to seven years. For example, the Center for Disease Control has estimated that there will about 300,000 AIDS cases by the 1991.



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HIV-1 also causes a somewhat less serious immunodeficiency syndrome clinically defined as AIDS related complex (ARC). ARC will often precede the onset of AIDS. There are currently many more ARC cases than there are AIDS cases. As the number of cases continues to increase, ARC will, in and of itself, become an extremely costly and serious health problem.

AIDS results because infection with HIV-1 damages and eventually destroys the victim's immune system. The immune system is reduced to the point where the victim can no longer ward off secondary opportunistic infections. It is often the secondary infections which debilitate the victim and cause death.

In addition to their susceptibility to secondary infections, AIDS victims frequently develop otherwise rare conditions. A large number develop a rare form of skin cancer known as Kaposi's sarcoma. It is believed that this condition also results from the immunodeficiency brought on by the virus.

HIV-1 damages the immune system by infecting and depleting T helper/inducer lymphocytes (hereinafter referred to "T cells"). T cells are essential because they control the production of antibodies by the B cells, the maturation of cytotoxic T lymphocytes (killer T cells), the maturation and activity of macrophages and natural killer cells, and, directly and indirectly, numerous other regulator and effector functions of the immune system.

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Infection of a T cell occurs through interaction between an epitope borne by HIV-1 and a receptor site which is located on the T cell surface. This receptor site on the T cell is protein molecule known as the CD4 antigen. The epitope on HIV-1 is borne by the envelope glycoprotein gp 120 (molecular weight 120,000 daltons). The glycoprotein gp 120 is produced when a precursor glycoprotein gp 160, made in the T cell, is cleaved apart into gp 41 (molecular weight 41,000 daltons) and gp 120. Gp 41 bears the epitope which induces the dominant antibody response in most infected individuals, whereas the epitope borne by gp 120 binds to the CD4 antigen and thereby allows the virus to enter the cell.

HIV-1 is a retrovirus. After the virus has entered the cell, a viral enzyme called reverse transcriptase transcribes the viral genomic RNA into DNA in the host cell nucleus. The newly synthesized DNA acts as a template and causes the infected T cell to begin to transcribe the new DNA to make copies of messenger RNA and genomic RNA. The viral genomic RNA's are packed with core proteins, reverse transcriptase, and certain other proteins. They are then enveloped by parts of the cellular membrane and budded off from the cell into the bloodstream as newly synthesized virions. These new virions can enter and infect other T cells.

There are two known mechanisms by which HIV-1 is transmitted to T cells in the body of infected individuals. The first occurs when the free virus

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binds to the CD4 antigen on the T cells. The second mechanism is through direct, cell-to-cell transmission of the virus.

Direct, cell-to-cell transmission occurs when an infected cell, which expresses the viral gp 120 on its surface, binds with the CD4 antigen of an uninfected cell. As a result the two cells fuse and virions can pass to the uninfected cell.

Direct, cell-to-cell contact and the resulting fusion are a significant source of cellular infection, and may be a major mechanism of T cell destruction in HIV-1 infected individuals. Infected and uninfected cells often fuse in large groups, thereby forming multi-nucleated aggregates known as syncytia. The cell fusion causes the death of cells in the syncytia. See Lifson et al. "Induction of CD4-Dependent Cell Fusion by the HTL-III/LAV Envelope Glycoprotein", Nature 323:725-27 (1986).

The majority of cell death is believed to take place in syncytia. This theory follows because it seems unlikely that significant infection can occur from other sources, such as free virus in the bloodstream. Concentrations of free virus in the bloodstream of infected individuals are typically very low. It also seems unlikely that significant cell infection can occur from discrete fusion of individual infected and uninfected cells. In one study it was found that the proportion of infected T cells in infected individuals is usually only one out of every 10,000 to 100,000 white blood cells.

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Nevertheless it was reported that the number of CD4 positive cells (i.e., T cells) gradually decreased.

Patients who are infected by HIV-1 do not generate sufficient amounts of neutralizing antibodies. They typically have very low titers of neutralizing antibodies in their serum. Thus, monoclonal antibodies which neutralize HIV-1 would be particularly useful for treatment.

Monoclonal antibodies are produced by hybridoma cells. Hybridomas are cells which have all been cloned from a single fused cell. All the clones are identical to the parent. Accordingly, all the hybridomas of the same clone produce identical antibodies which bind to the same epitope.

A method of making monoclonal antibodies was first described by Koehler and Milstein. See Milstein et al., Nature 256:495-97 (1975); Koehler et al., Eur J. Immunol., 6:511-19 (1976). A host animal, usually a mouse, is immunized with an antigen and then sacrificed. Lymphocytes containing B-cells are then removed, usually from the spleen or other lymphoid tissues. The removed lymphocytes are fused with myeloma cells to form hybridomas. The hybridomas which produce antibody against the designated epitopes of the immunizing antigen are cloned and screened. These hybridomas are then used to manufacture the desired monoclonal antibodies.

A monoclonal antibody which inhibits infectivity and syncytium formation would have many advantages over other neutralizing agents. Large quantities of the monoclonal antibody could be produced.

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The hybridomas are immortal due to the fusion with myeloma cells, and can be reproduced almost endlessly.

Another advantage of monoclonal methodology is that monoclonal antibodies of high specificity and high affinity can be screened from a large number of antibodies of diverse reactivities and affinities. If one can obtain antibody of high specificity and high affinity, this may allow therapeutic use of the antibody in minimal quantities which are just sufficient enough to bind the appropriate epitopes to neutralize the virus and to prevent syncytia formation.

The high specificity of monoclonal antibodies is to be contrasted with that of other neutralizing agents. In one study, antisera was collected from goats which had been immunized with various proteins from the envelope of HIV-1, including gp 120. The antisera effectively blocked infection of HIV-1 only at low dilutions. See S.D. Putney et al., "HTLV-III/LAV- Neutralizing Antibodies to an E. coli-Produced Fragment of the Virus Envelope", Science 234:1392-95 (1986). Similarly, antisera from rabbits and guinea pigs which were immunized with recombinant gp 120 was effective for HIV-1 neutralization only at low dilutions. See L.A. Lasky et al., "Neutralization of the AIDS Retrovirus by Antibodies to a Recombinant Envelope Glycoprotein", Science 233:209-212 (1986). The polyclonal antibodies used in these studies are non-specific and

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therefore had to be used in relatively large quantities.

The above results suggest that entire gp120 and long recombinant peptides can not induce high titer neutralization antibodies probably because the "neutralization epitopes" are not immunogenic. Moreover, the antibodies are found to be type-specific and not group-specific, i.e. they react with only the immunizing HIV-1 strain and not with other strains that are genetically significantly different.

In order for a monoclonal antibody to be used for therapeutical and prophylactic purposes in AIDS, it must exhibit protective activity against diverse HIV-1 strains and a large number or a significant proportion of field HIV-1 isolates.

In summary, a monoclonal antibody of potential therapeutical value to treat patients with AIDS or ARC and of protective value in preventing AIDS in asymptomatic healthy HIV-1 infected individuals or in preventing HIV-1 infection in individuals of high-risk groups is one that inhibits infection of susceptible cells by broad strains of HIV-1 either via attack by free virions or by direct cell-to-cell transmission (syncytium formation).

#### Summary of the Invention

Monoclonal antibodies which bind to the viral envelope glycoprotein gp 120 of HIV-1 have been isolated. The monoclonal antibodies inhibit HIV-1 infection of T cells by free virions, and they also

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inhibit syncytium formation. Importantly, the monoclonal antibodies are group specific and can neutralize and crossprotect against different strains and different isolates of HIV-1.

The HIV-1-neutralizing antibodies can be used for treatment of AIDS and ARC and for passive immunization against HIV-1 infection. In these procedures, the antibodies can be used as whole antibodies or as antibody fragments or they can be conjugated to cytotoxic or antiviral agents, or to microcarriers which contain such agents in order to target the delivery of these agents to infected cells. The targeted delivery of therapeutic agents can also be achieved with bispecific antibodies derived from the anti-HIV-1 antibodies of this invention which have been provided with a second specificity for the agent to be delivered to the target. Polyclonal or monoclonal antibodies against paratope of the neutralizing antibodies can be used to stimulate a neutralizing immune response against HIV-1.

The monoclonal antibodies of this invention can be used in vivo as antibodies derived wholly from mice or other animals. Alternatively, especially for therapeutic use, viral neutralizing antibodies can be made in the form of animal/human chimeric antibodies. Preferably, the constant region of the chimeric antibody is human-derived, and the variable region is animal-derived.

The monoclonal antibodies of this invention are produced by continuous, stable antibody-producing

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cell lines. These cell lines can be produced by hybridoma techniques and by genetic engineering techniques.

This invention also pertains to peptides which correspond to epitopic segments of gp120 recognized by the antibodies of this invention. The peptides can be used in vaccine compositions for generating a crossprotective, neutralizing immune response against HIV-1. They can also be used to detect neutralizing antibodies against HIV-1 in a biological fluid.

#### Brief Description of the Drawings

Figure 1 is a plot showing the relative effectiveness of four of the monoclonal antibodies of the invention in neutralizing HIV-1 infection of H9 cells. The percentage of infected cells was determined nine days after infection.

Figure 2 is a plot showing the relative effectiveness of four of the monoclonal antibodies of the invention in neutralizing HIV-1 infection of H9 cells. The percentage of infected cells was determined thirteen days after infection.

Figure 3 is a schematic depiction of the structure of chimeric genes encoding a light and a heavy chain for a chimeric HIV-neutralizing antibody. (A) Plasmid pSV184 $\Delta$ Hneo.BAT123V $_{\kappa}$ .hC $_{\kappa}$  contains a chimeric light chain gene construct consisting of a 4.4 kbp Hind III fragment of mouse V $_{\kappa}$  gene fused with the human C $_{\kappa}$  gene. This plasmid contains a neo selection marker. (B) Plasmid pSV2 $\Delta$ Hgpt.BAT123V $_{H}$ .



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hC<sub>γ1</sub> contains a chimeric heavy chain gene construct consisting of a 4.5 Kbp Eco RI fragment of mouse V<sub>H</sub> gene fused with the human C<sub>γ1</sub> gene. The plasmid carriers an Eco gpt selection marker. B:Bam HI, E:Eco RI, H:Hind III, S:Sal I, V:variable region gene, C:constant region.

Figure 4 shows isoelectric focusing patterns of the chimeric HIV-neutralizing antibody (lane 2) and the murine monoclonal antibody BAT123 (lane 1). S represents the pH calibration standard proteins with pI values of (from cathode (top) to anode (bottom)): 8.65, 8.45, 8.15, 7.35, 6.85, 6.55, 5.85, 5.20, 4.55, and 3.50.

Figure 5 shows electrophoretic analysis of the chimeric immunoglobulin. (A) Immunoglobulins purified by r-protein A affinity chromatography were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel with or without reducing the disulfide bonds. Lane 1, BAT123, reduced; lane 2, chimeric antibody, reduced; lane 3, BAT123, unreduced; lane 4, chimeric antibody, unreduced. (B) Western blot analysis of the immunoreactivity of the immunoglobulin to anti-mouse antibody. Purified immunoglobulin (2μg) were resolved in 10% SDS-PAGE under reducing conditions, electro-transblotted onto nitrocellulose membrane filter and reacted with anti-mouse antibody. Lane 1, BAT123, lane 2, chimeric antibody. (C) Duplicate transblotted membrane from (B) was reacted with anti-human antibody. Lane 1, BAT123; lane 2, chimeric antibody.

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Figure 6 shows the IgG subclass of the chimeric antibody as assayed by its reactivity to the anti-sera in ELISA. Anti-sera used were (●) anti-human IgG1, (○) anti-human IgG3.

Figure 7 shows Western blot analysis of the antigen specificity of the chimeric antibody. (A) Immunoreactivity of the immunoglobulins to HIV-1 antigens which were resolved in SDS-PAGE and trans-blotted onto nitrocellulose membrane filters. Strip 1, an AIDS patient serum (1:200 dilution), strip 2, BAT123 (1  $\mu$ g/ml); strip 3, chimeric antibody (1  $\mu$ g/ml). (B) Reactivity of the antibody to the synthetic oligopeptides representing potential antigenic determinants (epitopes) of HIV-gp120 which were blotted on nitrocellulose membrane filter. Strip 1, BAT123; strip 2, chimeric antibody.

Figure 8 shows the neutralizing activities of chimeric antibody on the infection of H9 cells by HIV-1. Cell-free culture supernatants were collected at day 14 for HIV-1 specific antigen capture assays after the H9 cells were challenged with HIV-1 in the absence and in the presence of antibody tested. Each concentration of the antibodies was tested in triplicate. The inhibition of HIV-1 infection was calculated by comparing the optical densities obtained in the antigen capture assays of cultures infected with the virus in the presence of antibody to the negative control without added antibody. The antibodies tested were BAT123 (○), Chimeric antibody cAG1-51-4 (●), and a non-reactive murine anti-hcG antibody (Δ).

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Detailed Description of the InventionA. Summary of Procedures Used

The monoclonal antibodies of the invention bind to the viral envelope glycoprotein gp 120. In the processing of HIV-1 specific envelope protein in infected T cells, gp 41 is a transmembrane domain and is not exposed. In contrast, gp 120 is an external envelope protein which is extracellular. Thus, in infected T cells the gp 120 protein offers binding epitopes for the monoclonal antibodies of the invention.

The monoclonal antibodies of the invention were found to be effective in inhibiting infectivity and in inhibiting syncytium formation. This indicates that they will likely be very effective for in vivo neutralization, as the majority of cell death is believed to occur in syncytium. Importantly, the antibodies can neutralize different strains and different isolates of HIV-1 (i.e. the antibodies are group specific). The antibodies neutralize infection of cells by different strains and different isolates of the virus. The neutralizing antibodies also inhibit syncytium formation by various strains of HIV-1 which have a substantial degree of heterogeneity in the amino acid sequence of gp 120. These results indicate that antibodies are cross-protective and are able to protect against the various strains of the virus in the population.

The neutralizing antibodies of this invention can have high potency in neutralizing infectivity. For example, the monoclonal antibodies can inhibit,

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with an  $IC_{50}$  of less than 10  $\mu$ g/ml, the infection of susceptible human T cells lines by HIV-1<sub>B</sub> at 20 times  $TCID_{50}$  in a nine day assay.

The general procedure for production of the antibodies is discussed below.

The monoclonal antibodies of the invention were made by conventional techniques which are commonly used in hybridoma production. In brief, mice were immunized with inactivated HIV-1. B cells taken from the spleens of the immunized mice were fused with NS-1 myeloma cells. Polyethylene glycol mixed with dimethyl sulfoxide (DMSO) in calcium magnesium-free phosphate buffered saline (PBS) was used as the fusion reagent. The hybridomas generated from the fusion were then transferred to 96 well microtiter plates and grown.

The hybridomas which produced the monoclonal antibodies that neutralized HIV-1 were isolated by a series of screening procedures. First, an enzyme linked immunosorbent assay (ELISA) was run on the clones in all the wells. In this test, it was determined whether monoclonal antibodies produced by these clones would bind to purified gp 120. Clones from those wells which showed highest reactivities with gp 120 were selected for further screening by an immunofluorescence assay.

The immunofluorescence assay was run to determine which of the ELISA positive monoclonal antibodies would bind specifically to intact, live infected T cells, but not to uninfected T cells. The clones found to be immunofluorescence positive,

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i.e., those which produced antibody specific to the infected cells, were used in a single-cell cloning.

In single-cell cloning the clones are diluted so that there are only a few cells per given volume. This volume is then added to a well, and the cells are grown. The objective is to attain randomly by binomial distribution in some wells only a single-cell colony per well. This cell colony is monitored visually under a microscope to determine whether it is a monoclonal.

The ELISA-positive clones were also tested in a Western blot analysis. In this procedure lysates of HIV-1 proteins were separated by gel electrophoresis and transferred onto nitrocellulose strips. The supernatants from the ELISA-positive wells are then tested for reactivity with gp 120 protein band on the strips.

At the conclusion of these screening steps, monoclonal antibodies which were specific to the gp 120 and to infected cells had been isolated.

The immunofluorescence-positive hybridomas were then injected into the peritoneum of mice for production of a larger quantity of monoclonal antibodies from the ascites fluid. The antibodies were then purified for assays for neutralization.

A number of modifications of the above immunization, fusion, screening, and method of antibody production are possible. For example, animals other than mice can be for the immunization. B cells are then obtained from the immunized animal for use in the fusion.

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Further, reagents other than those discussed can be used for the chemical fusion. Another alternative is to use electrical fusion rather than chemical fusion to form hybridomas. This technique is well-established. Instead of fusion one can also transform a B-cell to make it immortal using, for example, an Epstein Barr Virus or a transforming gene. (For a method of transforming a B-cell, See "Continuously Proliferating Human Cell Lines Synthesizing Antibody of Predetermined Specificity," Zurawaki, V.R. et al, in Monoclonal Antibodies, ed. by Kennett R.H. et al, Plenum Press, N.Y. 1980, pp 19-33.)

With regard to the ELISA, the immunofluorescence assay, and the Western Block analysis, it should be noted that several alternatives of all these steps are possible. One could do a greater or lesser number of screening steps. Or, instead of those which are described, one could substitute other screening procedures, for example, a radio-immunoassay, or an immunohistochemical staining techniques. The important consideration is that the procedure selects for hybridomas which secrete monoclonal antibodies which are specific for gp 120 and which specifically bind to the cell surface of HIV-1 infected T cells.

The single cell cloning procedure can be varied such that various numbers of cells initially are placed into each well. The test for whether there is in fact only one clone present in each well can also be performed by a number of methods.

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The method of producing monoclonal antibodies, i.e., injecting hybridomas into mice, can also be varied. It is possible to grow large quantities of monoclonal antibodies in culture using perfusion or hollow-fiber techniques.

After isolation of monoclonal antibodies specific to gp 120 and to intact, live infected cells by the above-described methods, the effectiveness of the antibodies in neutralization of HIV-1 was tested. Monoclonal antibodies from each clone which was immunofluorescence positive were isolated. A comparison was made of the number of cells infected by HIV-1 in the presence or absence of the monoclonal antibodies. Different titers of each antibody were used in order to compare their potency. The neutralization assay was monitored with an immunofluorescence technique.

The second test of neutralization is by syncytium inhibition. In the syncytium inhibition assay infected T cells were added to a well seeded with transfected HeLa cells which have been artificially transfected with CD4 genes and the CD4 antigen on their surface. The CD4 antigen on the cell surface fuses with infected T cells to form multi-nucleated giant cells. It was determined whether different titers of the immunofluorescence positive antibodies would inhibit giant cell formation.

A great deal of modification of the neutralization assays and their monitoring procedures is possible. It may be desirable to test only one and not the other if, for example, one was concerned

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only with syncytium formation and not with infection through free virion particles.

The antibodies are tested in these assays for neutralization of different viral strains and isolates.

In conclusion, a variety of ways of preparing and testing the products of the invention are possible and are within the scope of the invention. The advantages and uses for the monoclonal antibodies of the invention will now be discussed.

B. Advantages and Uses

As noted above, one advantage of monoclonal antibodies is their specificity. This specificity is highly pertinent to use of the monoclonal antibodies of the invention in therapy, because it means that lower dosages can be used.

The therapeutic uses for the monoclonal antibodies of the invention include both in vivo immunotherapy and extracorporeal immunotherapy. Direct in vivo treatment with the monoclonal antibodies of the invention involves administering them internally, preferably via intravenous injection. If treatment of infected cells in the brain is needed, it may be possible to couple the monoclonal antibody to an agent, such as certain lipophilic substances, which allows it to pass through the blood-brain barrier. The antibodies of this invention can neutralize different strains and isolates of HIV-1 and thus, they can effectively protect against the different



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types of virus encountered in the patient population.

In extracorporeal therapy, blood leukocytes are removed from the patient and treated with neutralizing antibody. The monoclonal antibody is then added to the leukocytes. The leukocytes can also be stimulated with immunopotentiating drugs, for example interleukin-2. The leukocytes are then returned to the patient.

The mouse-derived monoclonal antibodies of the invention can be used for both direct in vivo and extracorporeal immunotherapy. However, it has been observed that when mouse-derived monoclonal antibodies are used in humans as therapeutic agents, the patient produces human anti-mouse antibodies. Thus, it has been said that mouse-derived monoclonal antibodies, at least in some cases, have limited therapeutic value. See V.T. Oi et al., "Chimeric Antibodies," Bio Techniques 4(3):214-221 (1986). With established genetic engineering techniques it is possible, however, to create antibodies which have animal-derived and human-derived portions. Chimeric antibodies comprise an antigen-binding (variable) region derived from an animal antibody and a constant region derived from a human antibody. Production of viral neutralizing antibodies are described below.

Another alternative form of monoclonal antibody is a bispecific antibody. Bispecific antibodies carry two different antigen binding portions, both of different specificity. A bispecific monoclonal

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antibody can have one antigen binding portion derived from the monoclonal antibodies of the invention, and a second antigen binding portion of a specificity for an agent to be targeted to a particular site. For example, the second specificity can be for a surface epitope of a human T cell or of a macrophage, such as the CD4 molecule. These bispecific antibodies can be used to target a T cell or macrophage toward an HIV-1 infected cell.

The bispecific antibodies can be single, hybrid antibodies or antibody fragments having a bispecificity (See M. Brennan, "A Chemical Technique for the Preparation of Bispecific Antibodies from Fab' Fragments of Mouse Monoclonal IgG<sub>1</sub>", Biotechniques 4:424-27 (1986)) or they can be heteroaggregates of two antibodies each having a different specificity.

The potential patient populations for receiving immunotherapy antibody treatments include patients with AIDS or ARC. A variant of immunotherapy is protection through passive immunization. The antibodies of this invention are particularly suitable for passive immunization because they can crossprotect against HIV-1 of different strains in the population. In this procedure, patients who are asymptomatic (not yet showing symptoms of AIDS or ARC), or who are seronegative but in a high risk group, are treated to inhibit infection. The targets include fetuses born in or babies born to HIV-1-carrier mothers and health professionals working with AIDS patients, or blood products, such

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as dentists and nurses. The agent for treatment, again, can be the monoclonal antibodies of the invention, chimeric mouse/human monoclonal antibodies, or bispecific monoclonal antibodies.

Most of the research attention in the effort to stop AIDS has focused on the search for a vaccine. In one type of proposed vaccine the immunizing agent is a portion of HIV-1 which itself is non-infective but which nonetheless induces antibody production. Monoclonal antibodies which neutralize HIV-1 can help in the search for such a vaccine. They can be used to help locate, identify, and study the "neutralizing" epitopes on HIV-1 which bind the monoclonal antibodies. These epitopes are likely to be the non-infective but nonetheless immunogenic portion of the molecule. Study of these epitopes allows synthesis of a non-pathogenic immunogen with a structure which is the same or immunologically equivalent to the epitope. For example, the immunogen can be a peptide which comprises an amino acid sequence that is the same or similar to the epitope bound by an anti-HIV-1 antibody which neutralizes HIV-1.

It has now been discovered that two of the neutralizing antibodies of this invention recognize epitopes located in a region of gp 120 having the following amino acid sequence:

RPNNNTRKSIRIQRGPGRAFVTICK

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This segment represents a 25 amino acid residue long segment of gp 120 (residue # 284 to residue # 308). One antibody (BAT267) reacts with a peptide having the sequence RPNNNTRKSIRIQRG (peptide a) and the other antibody (BAT123) reacts with a peptide having the sequence RIQRGPGRAFTIGK (peptide b).

These two 15 amino acid residue long peptides represent two adjacent, overlapping segments of gp120 of HIV-1B: peptide "a" represents the segment of residue #294 to residue #308 and peptide "b" of residue #304 to #318. BAT267 reacts with peptide "a" and not peptide "b", which shares five amino acids RIQRG, or another 15 amino acid long peptide, which represents a segment of gp120 (residues #284 to #298) adjacent to peptide "a" and shares five amino acids RPNNN. These results suggest that BAT267 recognizes an epitope either borne entirely by all or a part of the middle five amino acid residues TRKSI or formed by all or a part of these five amino acids with some of the flanking amino acid residues. Based on similar results, BAT123 seems to react with an epitope either borne entirely by all or a part of PGRAF or formed by the combination of all or a part of PGRAF with some of the flanking amino acid residues.

The BAT085 antibody reacts with a peptide having the amino acid sequence VQKEYAFFYKLDIIP.

The peptidic immunogens of this invention can comprise the above-identified amino acid sequences or immunochemical and immunogenic equivalents thereof. These equivalents include, for example,

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any of the actual epitope portions of any of these sequences, corresponding peptidic regions from various HIV-1 strains and peptides generated by various changes such as insertions, deletions and substitutions of amino acids.

The peptides of this invention can be coupled together to form larger, multivalent oligopeptides.

The peptides may be prepared by chemical synthesis. Alternatively, they may be prepared by recombinant DNA technology where DNA sequences encoding the peptides are synthesized or isolated from HIV-1 DNA and expressed in an appropriate expression system.

The peptides may also be used individually or in combination to elicit a immune response against HIV-1. For this purpose, the peptides may be formulated in vaccine compositions, generally for administration at concentrations in the range of 1 ug to 20 mg/kg of host. Physiologically acceptable vehicles such as water, saline, or phosphate buffered saline can be used in the formulations. Adjuvants, such as aluminum hydroxide gel, can also be employed. The route of administration can be intramuscular, intraperitoneal, subcutaneous, or intravenous. The compositions can be given one time or multiple times, usually at one to four week intervals.

In preferred embodiments of the vaccine composition, the peptides are coupled to a carrier protein such as a foreign keyhole limpet hemocyanin.

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This can enhance the immunogenicity of the haptenic peptides.

The peptides may be used in immunoassays to identify neutralizing antibody or to screen for the presence of neutralizing antibody in serum.

Another type of vaccine which monoclonal antibodies that neutralize HIV-1 make possible is one based on an anti-idiotypic antibody. Antibodies carry "idiotypes", regions near their antigen-recognition sites that are themselves antigenic and capable stimulating antibody production. Antibodies which are specific to the antigen-combining sites are called paratope-specific anti-idiotypic antibodies. These antibodies bear the same confirmation as the antigen which initially stimulated antibody production. See J.L. Marx, "Making Antibodies Without Antigens", Science 288:162-65 (1986).

Thus, paratope-specific anti-idiotypic antibody with partially the same structure as HIV-1 can be made by immunizing an animal with the monoclonal antibody to HIV-1. These paratope-specific anti-idiotypic antibodies, which carry certain same structure as the immunogenic portions of the virus, would likely be suitable for use as a vaccine because they would cause an immune response. Advantageously, because these anti-idiotypic antibodies consist of protein and do not carry any viral nucleic acid, they would be much less concern for pathogenicity. A chimeric mouse/human anti-idiotypic antibody wherein the variable region is mouse

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monoclonal anti-idiotypic antibody and the constant region is human immunoglobulin, is most preferable.

The monoclonal antibodies of the invention could also be used to aid in the delivery of cytotoxic or antiviral agents, by incorporating them into, for example, microcarriers or liposomes. Exemplary cytotoxic agents include cytotoxic steroids, galanin, abrin, ricin and phospholipases. Examples of antiviral agents are interferon, azidothymidine and ribovirin. Once again, it should be noted that chimeric mouse/human monoclonal antibodies, or bispecific monoclonal antibodies, are also suited to aid in drug delivery.

In the conventional sense, antibodies, including monoclonal antibodies, are referred to as the mediator of humoral immunity. However, because antibodies which are specific for unique cell surface antigens on target cells can be conjugated with cytolytic or cytotoxic agents, the resulting immunotoxins can in effect mediate cellular immunity. Cytotoxic T lymphocytes, which are the key mediator of antigen-specific cellular immunity, recognize and lyse viral-infected cells. Thus, with proper engineering, the antibodies specific to viral antigenic epitopes expressed on infected cell surface can achieve the major function of cytotoxic T lymphocytes.

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C. Production of Chimeric Viral-Neutralizing Antibodies (Immunoglobulins)

Chimeric viral-neutralizing immunoglobulins of this invention are made up of chimeric heavy and light immunoglobulin chains. Each chimeric chain is a contiguous polypeptide that has a nonhuman variable region and a human constant region. The chimeric heavy and light chains are associated to form a molecule with a functional antigen binding region.

The chimeric immunoglobulins can be monovalent, divalent or polyvalent. Monovalent immunoglobulins are dimers (HL) formed of a chimeric heavy chain (H) associated (through disulfide bridges) with a chimeric light chain (L). Divalent immunoglobulins are tetramers ( $H_2L_2$ ) formed of two associated dimers. Polyvalent antibodies can be produced, for example, by employing heavy chain constant region which aggregate (e.g., mu type constant regions).

The chimeric immunoglobulins can be produced as antigen binding fragments. Fragments such as Fv, Fab, Fab' or  $F(ab')_2$  can be produced by employing appropriately truncated heavy chain constant regions.

The variable regions of the chimeric immunoglobulins are derived from nonhuman immunoglobulins having the desired viral specificity and viral-neutralizing properties. In preferred embodiments, the parent antiviral immunoglobulin neutralize different types, strains and isolates of a virus. This provides crossprotection against viruses of different types, stains and isolates that are encountered in viral populations.



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Important pathogenic viruses for which chimeric viral-neutralizing antibodies can be produced are HIV, human T cell lymphotropic virus I (a causative agent of adult T cell leukemia), and hepatitis B virus.

HIV-neutralizing immunoglobulins are described above. These HIV-neutralizing antibodies specifically react with the glycoprotein gp120 of HIV-1; they inhibit the infection of T cells by free virions and inhibit infection of T cells by fusion with HIV-infected cells. The antibodies are preferred because they are cross-neutralizing, i.e. they neutralize different strains and isolates of HIV-1. For example, antibody BAT123 is especially preferred because of its neutralizing activity and cross strain reactivity. The BAT123 antibody inhibits with an  $IC_{50}$  of less than 10 ng/ml, the infection of a susceptible human T cell line, H9, by HTLV-III B strains at 20 times  $TCID_{50}$  in a nine day assay. The antibody also inhibits some other cloned HIV-1 strains and it inhibits the in vitro replication of broad, freshly isolated field HIV-1 samples from patients.

The heavy chain constant region for the chimeric immunoglobulins can be selected from any of the five isotypes alpha, delta, epsilon, gamma or mu. Heavy chains of various subclasses (such as the IgG subclasses 1-4) can be used. The different classes and subclasses of heavy chains are involved in different effector functions and thus, by choosing the type of heavy chain constant region,

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chimeric antibodies with desired effector function can be produced. The light chains can have either a kappa or lambda constant chain.

The chimeric immunoglobulins of this invention are produced by genetic engineering techniques. Appropriate recipient cells are transfected with nucleic acid constructs, preferably DNA, encoding the desired chimeric light or heavy chain. In general, DNA constructs for each of the light and heavy chain components of the chimeric immunoglobulin comprise a fused gene comprising a first DNA segment which encodes at least the functional portion of the variable region linked to a second DNA segment encoding at least a part of a constant region. The fused gene is assembled in or inserted into an expression vector for transfection of the appropriate recipient cells.

In preferred embodiments the fused gene construct will comprise a functionally rearranged gene encoding a variable region of a chain of a viral-neutralizing immunoglobulin linked to a gene encoding a constant region of an immunoglobulin chain. The construct will also include the endogenous promoter and enhancer for the variable region encoding gene. For example, the variable region encoding genes can be obtained as DNA fragments comprising the leader peptide, the VJ gene (functionally rearranged variable (V) regions with joining (J) segment) for the light chain or VDJ gene for heavy chain, and the endogenous promoter and enhancer for these genes. These variable region

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genes can be obtained from antibody-producing cells that produce the desired viral-neutralizing antibody by standard DNA cloning procedures. See Molecular Cloning: A Laboratory Manual. T. Maniatis et al.. Cold Spring Harbor Laboratory (1982). Screening of the genomic library for the functionally rearranged variable region can be accomplished with the use of appropriate DNA probes such as DNA segments containing the mouse germline J region DNA sequences and sequences downstream. Identification and confirmation of the correct clones are then achieved by DNA sequencing of the cloned genes and comparison of the sequence to the corresponding sequence of the full length, properly spliced mRNA. The DNA fragment containing the functionally rearranged variable region gene is linked to a DNA fragment containing the gene encoding the desired constant region (or a portion thereof).

Genes encoding antibody light and heavy chains can be obtained generally from immunoglobulin-producing lymphoid cells. Hybridoma cell lines producing antibody against a desired virus can be made by standard procedures. See, Koprowski et al.; U.S. Patent No. 4,196,265. In general, these entail challenging a animal with a virus or a purified or partially purified viral antigen, fusing antibody-producing cells taken from the immunized animal with compatible myeloma cells to form hybridoma cells, cloning the resulting hybridoma cells and selecting clones which produce antibody against the virus. The hybridoma clones can be screened for the

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production of viral-neutralizing antibody by tests for neutralizing activity for the particular virus. For example, several in vitro assays for HIV-neutralizing activity are described herein.

Human constant regions can be obtained from antibody-producing cells by standard gene-cloning techniques. Genes for the two classes of human light chains and the five classes of human heavy chains have been cloned, and thus, constant regions of human origin are readily available from these clones. Chimeric immunoglobulin fragments such as the monovalent Fv, Fab or Fab' fragments or the divalent F(ab')<sub>2</sub> fragment can be prepared by designing a chimeric heavy chain gene in truncated form. For example, a chimeric gene encoding a F(ab')<sub>2</sub> heavy chain would include DNA sequences encoding the CH<sub>1</sub> domain and at least sulfhydryl-containing part of the hinge region of the heavy chain.

The fused genes encoding either the light or heavy chains are assembled or inserted into expression vectors for incorporation into a recipient cell. Suitable vectors for the chimeric gene constructs include plasmids of the types pBR322, pEMBL, and pUC. The introduction of gene constructs into plasmid vectors can be accomplished by standard procedures.

In preferred embodiments, the expression vector is designed to contain two selectable genetic markers, one for selection in a prokaryotic (bacterial) system and the other is for selection in a eukaryotic system. The fused genes can be produced

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and amplified in a bacterial system and subsequently incorporated and selected for in eukaryotic cells. Examples of selectable genes for a prokaryotic system are the gene which confers ampicillin resistance and the gene which confers chloramphenicol resistance. Two genes for selection of eukaryotic transfectants are preferred (i) the xanthine-guanine phosphoribosyl-transferase gene (designated gpt) and (ii) the phosphotransferase gene from Tn5 (designated neo). Selection with gpt is based on the ability of the enzyme encoded by this gene to use xanthine as a substrate for purine nucleotide synthesis; the analogous endogenous enzyme cannot. In a medium containing xanthine and mycophenolic acid which blocks the conversion of inosine monophosphate to xanthine monophosphate, only cells expressing the gpt gene can survive. The product of the neo blocks the inhibition of protein synthesis in eukaryotic cells caused by the antibiotic G418 and other antibiotics of its class.

The chimeric light and heavy chain genes can be placed in two different expression vectors which can be used to cotransfect a recipient cell. In this case, each vector is designed to have a different selectable gene for eukaryotic transfectants. This allows cotransfection of the recipient cell and selection of cotransfected cells (i.e. cells that have received both vectors). Selection of cotransfected cells is accomplished by selection for both selectable markers, which can be done simultaneously or sequentially.

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Recipient cell lines are generally lymphoid cells. The preferred recipient cell is a myeloma. Myelomas can synthesize, assemble and secrete immunoglobulins encoded by transfected genes and they can glycosylate protein. A particularly preferred recipient cell is the myeloma Sp2/0 which normally does not produce endogenous immunoglobulin. When transfected, the cell will produce only immunoglobulin encoded by the transfected gene constructs. Transfected myelomas can be grown in culture or in the peritoneum of mice where secreted immunoglobulin can be recovered from ascites fluid. Other lymphoid cells such as B lymphocytes or hybridomas can be used as recipient cells.

There are several methods for transfecting lymphoid cells with vectors containing chimeric L and H chain genes. A preferred way of introducing a vector into lymphoid cells is the calcium phosphate precipitation procedure described by Graham and van der Eb, (1973) Virology 52:456. Another way is by electroporation. In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated into the cell. See e.g., Potter, et al. (1984) PNAS 81:7161. Another way to introduce DNA is by protoplast fusion. A lysozyme is used to digest cell walls from bacteria which contain the recombinant vector with the chimeric chain gene to produce spheroplasts. The spheroplasts are fused with the lymphoid cells in the presence of polyethylene glycol. After protoplast fusion, the transfectants are selected and

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isolated. (Oi, et al., (1983) PNAS 80:825). Finally, the DEAE-dextran procedure described by Cullen, et al., (1984) Nature 307:241 can also be used.

The chimeric viral-neutralizing immunoglobulins of this invention are useful for antiviral therapy and prophylaxis. Direct in vivo treatment with the chimeric immunoglobulins of this invention entails administering them internally preferably by intravenous injection in a pharmaceutically acceptable vehicle such as sterile saline. The antibodies can be administered in conjunction with other anti-viral agents.

A variant of immunotherapy is protection through passive immunization. In this mode, the antibody is administered to persons at risk of contracting viral infection in order to protect against infection.

Chimeric HIV-neutralizing immunoglobulins can be used to treat AIDS patients or persons who are HIV carriers. As mentioned above, chimeric immunoglobulins (of this invention formed, for example, from HIV-neutralizing immunoglobulins such as BAT123) are capable of neutralizing different strains and isolates of HIV-1. Further, these immunoglobulins can inhibit transmission of the virus by syncytia formation. The chimeric immunoglobulins can be administered to reduce viral load in an AIDS patient and to retard further progression of the disease. The immunoglobulins can be administered in conjunction with other anti-AIDS

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agents such as AZT. In addition, several different chimeric HIV-neutralizing immunoglobulins can be administered together.

In certain patient populations, passive immunization with the chimeric HIV-neutralizing immunoglobulins may be appropriate. In this procedure, patients who are asymptomatic (not yet showing symptoms of AIDS or ARC), or who are seronegative but in a high risk group, are treated to inhibit infection. The targets include fetuses carried by or babies born to HIV-1-carrier mothers and health professionals working with AIDS patients, or blood products, such as dentists and nurses.

The invention is illustrated further by the following exemplification.

Example I: Preparation of the Hybridomas and Monoclonal Antibodies

a) Preparation of Virus

In order to maintain a supply of inactivated HIV-1, a virus stock was prepared as follows. The H9 clones of the HT cell line (which is described by M. Robert-Guroff et al. in Nature 316:72-74, supra) were maintained in culture. These H9 cells were infected with HIV-1 (HTLV III<sub>B</sub>), which was a gift from Dr. R. Ting, Biotech Research Laboratory, Rockville, Maryland. Maintaining the infected H9 cells in culture permits the cells to reproduce and to continuously synthesize a supply of HIV-1. The H9 cells were cultured in a growth medium of 20% FBS (heat-inactivated) RPMI 1640, supplemented with 5mM



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L-glutamine, 5mM HEPES, 50 units/ml penicillin and 50 mg/ml streptomycin.

Purified HIV-1 was obtained by first centrifuging the cell culture at 1000 g for ten minutes to remove the cells and debris. The supernatant was then centrifuged at 90,000 g for one hour. The virus pellet was resuspended in minimal volume of phosphate buffered saline pH 7.4 and loaded onto a centrifuge tube with a preformed sucrose gradient (20%-60%). The sample was then centrifuged at 100,000 g for sixteen hours. The virus was collected at the gradient of 38%. The virus was then aliquoted and frozen at -80°C after the protein content was measured.

b) Immunization Procedure

Male Balb/c mice were used for the immunization. Each mouse received 100 µg of inactivated HIV-1. The inactivation of the virus was performed according to FDA approved protocol, by UV irradiation and addition of a detergent, Nonidet P-40 (0.1%). A volume of suspension containing 100 µg of virus per mouse was suspended in 200 µl phosphate buffered saline (PBS), and emulsified with equal volumes of complete Freund's adjuvant.

Each mouse was immunized subcutaneously with 100 µg of the emulsified virus. The mice were injected at sites with high concentrations of lymph nodes, for example, the underside of the inter-section of the limbs and the trunk. One month later the mice received subcutaneous booster injections at

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the same sites with the same quantity of virus. The boosters were prepared essentially in the same manner as was the first injection, except that for the boosters the emulsification was done in incomplete Freund's adjuvant.

One month later, each mouse was reimmunized subcutaneously with 100  $\mu$ g of virus suspended in PBS. Each mouse was injected subcutaneously at the intersection of each limb with the trunk, and intraperitoneally. Three days after the last injection, the mice were sacrificed and their spleens were removed. The spleen cells were then fused with myeloma cells by the following procedure.

c) Fusion

Suspensions containing a five-to-one ratio of spleen cells to myeloma cells were prepared. The myeloma cells chosen were NS-1. The NS-1 cells were conditioned to have a doubling time about every seventeen hours. They were used for fusion when in the log phase. The NS-1 cells were subcultured in bacteriological plates (100 mm) at a concentration of  $6 \times 10^4$  cells/ml in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 5% Fetal Bovine Serum (FBS), 100 units/ml of penicillin and 100 micrograms/ml of streptomycin. The medium was changed every three days. Alternatively, the cells were subcultured at  $1.54 \times 10^5$  cells/ml in 10 ml of the same medium, and the medium was changed every two days.

The spleen cells were prepared by placing the spleen on a bacteriological plate (100 mm) and injecting 20 ml of calcium magnesium free PBS

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(CMF-PBS) into both ends of the spleen to flush out the spleen cells. The flushed spleen cells were then transferred to a 50 ml centrifuge tube.

The spleen cells were centrifuged at 400 g for five minutes, and then suspended in 5 ml of 0.83%  $\text{NH}_4\text{Cl}$  (0.155 M) for ten minutes at room temperature to lyse the erythrocytes. 5 ml of CMF-PBS was added to the tube to stop the lysis. The cells were then pelleted, and resuspended in 10 ml of CMF-PBS.

The concentration of lymphocytes was determined by adding 40 microliters of cell suspension to 10 ml of saline together with 3 drops of Zap-oglobin<sup>TM</sup>. The number of lymphocytes was counted with a hemacytometer and from this value the concentration of cells was determined. The concentration was then multiplied by the dilution factor of 250 to yield the actual concentration of cells in the suspension.

The NS-1 cells were transferred from five of the bacteriological plates (100 mm) to a 50 ml centrifuge tube. The cell concentration was determined using the counting technique described above.  $5 \times 10^7$  of the NS-1 cells were then suspended in 10 ml of CMF-PBS and mixed with  $2.5 \times 10^8$  spleen cells in a 50 ml centrifuge tube.

The cells were spun down and washed once with 10 ml of CMF-PBS. The supernatant was aspirated as much as possible with a glass Pasteur pipette. The tube was gently tapped to free the cell pellet.

Prior to preparing the cells, a fusion mixture had been prepared as follows. 5 g of polyethylene glycol 1450 (purchased from Kodak) had been mixed

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The concentration of the cell suspension was adjusted to  $3.3 \times 10^5$  of the spleen cells per 200 microliters of suspension. 200 microliter aliquots of suspension were then distributed to each well of a 96 well microtiter plate. After seventeen such plates were prepared, the plates were transferred to an incubator and maintained at  $37^\circ\text{C}$  in  $5\% \text{CO}_2$ .

The cells were grown for seven days in the plates, then the growth medium was withdrawn and new medium was added. Four days after that, the medium was again changed. Four days later, an enzyme linked immunosorbent assay (ELISA) was performed on the antibodies in the wells to determine which would bind the gp 120 protein of HIV-1. The ELISA was carried out as follows.

d) ELISA Procedure

Purified gp 120 protein was prepared as described in W.G. Robey, "Prospect for Prevention of Human Immunodeficiency Virus Infection: Purified 120-kD Envelope Glycoprotein Induces Neutralizing Antibody", Proc. Natl. Acad. Sci. USA 83:7023-27 (1986). 50  $\mu\text{l}$  of a gp 120 suspension (at a concentration of 0.1 to 1.0  $\mu\text{g}/\text{ml}$ ) was added to wells of 96-well Immulon I plates with a twelve-channel pipette. The plates were covered and incubated for eighteen hours at  $4^\circ\text{C}$ , in order to allow the protein to bind to the plate.

The liquid contents of the plates were then emptied, and 200  $\mu\text{l}$  of 0.1 M  $\text{NH}_4\text{Cl}$  was added to each well in order to saturate any remaining binding

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with 5 ml of CMF-PBS and 0.5 ml of DMSO. This mixture had then been warmed to 56° C to melt it, titrated to a final pH of 7.0, and filtered through a 0.22 micron Millipore filter in order to sterilize it. 1.0 ml aliquots had been added to Cryotubes, and these had been stored at -70°C.

To prepare the fusion mixture for use, one of the aliquots in the Cryotubes was melted by heating it to 37°C. Separately, a tube containing 1- ml of DMEM (without serum) was heated to 37°C.

The 1.0 ml aliquot of polyethylene glycol fusion mixture was added to the cell suspension and the suspension was mixed well. Forty-five seconds after the polyethylene glycol fusion mixture had been added, 2.0 ml of the pre-heated DMEM (without serum) was added dropwise with mixing. The remaining 8 ml of the pre-heated DMEM (without serum) was then added. The cells were left at room temperature for 10 minutes.

2.0 ml of FBS was added to the suspension and the suspensions were mixed well. The combination of the FBS and the DMB-PBS can help to prevent adherence of cells to the test tube walls. The suspensions were then centrifuged at 400 g for four minutes.

After having been spun down, the cells were suspended in 116 ml of a modified medium, supplemented with 5% FBS, 100 units/ml of penicillin, 100 micrograms/ml of streptomycin, and Littlefield's hypoxanthine, aminopterin and thymidine (HAT).

sites on the plates. The  $\text{NH}_4\text{Cl}$  solution was left in the wells for thirty minutes at room temperature.

The  $\text{NH}_4\text{Cl}$  solution was then removed and the wells were washed three times with PBS and 0.05% Tween 20. Some of the PBS/0.05% Tween 20 solution was left in the wells until the antibody suspension described below was added.

50  $\mu\text{l}$  of the cell fusion supernatant from each well of the seventeen 96 well plates was added to each of the wells on the Immulon I plates, and incubated for one hour. Following incubation, the plates were rinsed three times with PBS/0.05% Tween 20 in order to remove any unbound antibody.

The cell fusion supernatant will contain the antibody which is produced by the various hybridomas in the 96 well plates. The antibody which is specific to gp 120 will bind thereto. Inasmuch as the gp 120 is bound to the Immulon I plate, the antibody specific to gp 120 will also become bound to the plate.

The next stage is to add the marker which will indicate the amount of bound antibody in each well. The marker chosen was horseradish peroxidase. This marker was conjugated with goat anti-mouse IgG to yield peroxidase-conjugated goat anti-mouse IgG. The goat anti-mouse IgG will bind to any mouse monoclonal antibody which is bound to the plate. the peroxidase marker can then be activated to indicate the quantity of bound antibody by an enzyme reaction.

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The marker was added by adding to each well 100 microliters of the peroxidase-conjugated goat anti-mouse IgG diluted at 1:1000 in PBS/0.05% Tween 20 and 1% BSA. The plates were incubated for one hour at room temperature. Thereafter, the plates were washed three times with PBS/0.05% Tween 20 to remove any unbound goat anti-mouse IgG conjugate.

The next step is to activate the peroxidase marker which is conjugated to the goat anti-mouse IgG. This is done by adding 200 microliters of 3', 3', 5', 5' tetramethyl benzidine substrate solution to each well, and incubating at room temperature for 30 minutes. The color reaction is stopped by adding 50 microliters of 2.0 M  $H_2SO_4$ .

The intensity of color was determined with an ELISA reader at 450 nm. The amount of antibody specific to gp 120 is proportional to the intensity of the color.

It was found that there were approximately 200 wells in the 96 well microtiter plates which produced antibodies which bound to gp 120 to at least some extent. Of these 200 wells the 39 which produced antibody showing the highest color intensity were selected for another screening step.

e) Immunofluorescence Assay Using Live T-Cells

An immunofluorescence assay was performed to determine whether any of the antibodies which were reactive with gp 120 in the ELISA would bind specifically to live HIV-1 infected H9 cells. The H9 cell line is permissive to persistent infection by HIV-1. This cell line was obtained from the

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American Type culture Collection in Rockville, Maryland. Antibody which binds to infected cells, but not uninfected cells, is probably selective to a domain of the HIV-1 envelope protein on the extracellular side of the cell membrane. The immunofluorescence assay helps to select those gp 120 reactive antibodies which have a high potential to recognize the neutralization epitopes on the HIV-1 virion, and to inhibit syncytium formation by infected T-cells.

Cultures of infected H9 cells were maintained as described above under the heading "Preparation of Virus". The procedure by which the assay was performed is described below.

(i) Assay Procedure

50  $\mu$ l aliquots of infected H9 cell suspension at a concentration of  $5 \times 10^6$  cells/ml was added to each of thirty-nine 1.5 ml microfuge tubes. 50  $\mu$ l aliquots of the supernatant from the 39 wells containing the ELISA positive clones was then added to each tube. The antibodies in the supernatant which react with H9 cells will bind to any H9 cells which are in the tube.

The tubes were then incubated for thirty minutes at room temperature. After incubation, the tubes were spun, the supernatant was withdrawn, and the cells were washed three times with a mixture of RPMI 1640, containing 2% fetal calf serum and 0.1% sodium azide. The tubes were then tapped to loosen the cell pellet.



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10  $\mu$ l of labeled antibody, goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC), was added to each test tube at a dilution of 1 to 200. This labeled antibody will bind to any monoclonal antibodies which have attached to HIV-1 infected H9 cells and provide a means for identifying these monoclonal antibodies.

The tubes were again incubated for thirty minutes at room temperature. The tubes were centrifuged, and the cells were washed with the same medium as before. The cells were then resuspended in PBS, placed onto individual slides and cover-slipped. The cells were viewed with a fluorescence microscope.

To determine which of the thirty-nine selected wells contained antibodies which bound to uninfected H9 cells, an essentially identical procedure as described above was performed, using infected H9 cells instead.

(ii) Results

Seven of the thirty-nine wells tested contained clones which produced monoclonal antibodies binding to live infected H9 cells but not to uninfected H9 cells. That is, when using antibodies from these seven wells the infected cells fluoresced, but the uninfected cells did not.

Cells and antibodies from the seven wells which contained immunofluorescence positive clones were collected. These hybridomas and antibodies have been deposited at the American Type Culture Collection in Rockville, Maryland, and are available

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for inspection by the Patent and Trademark Office during the pendency of this application.

f) Single Cell Cloning

Cell suspensions from each of the thirty-nine ELISA positive wells were expanded in the wells of a twenty-four well plate. After five days of growth in the twenty-four well plate, the cell suspension from the seven wells tested immunoreactive to infected H9 cells which were diluted to thirty, fifty and one hundred cells per milliliter. 0.1 ml of the diluted cell suspensions (containing an average of three, five and ten clones, respectively) was placed into the wells of a nine-six well plate. The wells had previously been coated with histone.

After each cell grew up to become a colony, the cells were checked under a microscope. The cells of each colony did not move about and form satellite colonies. The single-cell clone from each of the seven clonings showing strongest reactivities in ELISA and immunofluorescence was chosen and expanded in culture.

g) Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Procedure

In Western blot analysis, the virus is solubilized into its component proteins. The clones which produce monoclonal antibodies binding to the exterior envelope protein of HIV-1 (gp 120) are the ones which are desired. The procedure is described below.

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30 micrograms of HIV-1 was solubilized by heating it in a sample buffer (which contained 2% SDS and 5% beta-mercaptoethanol) at 100°C for five minutes. It was then loaded onto a 12% slab polyacrylamide gels 1.5 mm thick. The gel was run at constant voltage of 35 mV for 8 hours at room temperature. The procedure was described in "Procedure for Preparation of Gels for Western Blot Detection of HTLV-III Antibodies", published by Biotech Research Laboratories, Inc., Rockville, Maryland. The protein bands were transferred onto nitrocellulose paper by setting the power at 30 volts (about 0.1A) and running for 16 hours at room temperature. The next morning, the voltage was increased to 60 volts (about 0.2A) and the transfer was run for 1-2 hours to maximize the transfer of gp 120 and gp 160. The transfer buffer contained 24 g of Tris base, 57.6 g of glycine and 800 ml of methanol. Water was added to make the solution up to 4 liters.

The nitrocellulose sheets were then rinsed with PBS/0.05% Tween 20 and placed in a tray containing Blotto buffer. The tray was gently shaken for two hours at room temperature. Blotto buffer consists of 50 g of non-fat dry milk, 1.0 g of antifoam A (optional), 0.1 g of merthiolate, and sufficient PBS to make a final volume of 1.0 liter. The buffer pH was adjusted to 7.0.

The nitrocellulose sheets were then rinsed in PBS/0.05% Tween 20 and dried on a paper towel between weighted plexiglass plates. The

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nitrocellulose sheets were then cut into strips 0.5 cm wide, each of which was numbered consecutively. The strips can either be used immediately or stored dry and in the dark for up to one month. The strips which carry the gp 120 band were to be used in the next stage.

The gp 120 nitrocellulose strips were then prepared to allow binding of monoclonal antibody to the protein bands. Forty of these strips were individually placed into an assigned slot of a slot tray and pre-soaked for twenty minutes in PBS/0.3% Tween 20. The pre-soak solution was aspirated into a Clorox<sup>TM</sup> containing trap. The strip wells was then rinsed once with PBS/0.05% Tween 20, the tray was shaken several times, and the solution was aspirated off.

The positive control was made of 2.0 ml of Blotto buffer/4% goat serum (which is made by mixing 100 ml of Blotto buffer and 4 ml of heat inactivated normal goat serum) added to one strip after which 10 microliters of heat inactivated AIDS patient serum was added to the well. 2.0 ml of supernatant was withdrawn from each of the thirty-nine wells in the microtiter plates which contained ELISA positive clones. Mixtures were made which consisted of 2.0 ml of supernatant, 5% non-fat dry milk, 50 microliters of 1 M HEPES (pH 8.0), and merthiolate.

An aliquot of suspension was added into each strip well containing a strip. The mixture was then aspirated into a Clorox<sup>TM</sup> containing trap. The strips wells were rinsed once with PBS/0.05% Tween

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20, rocked several times by hand, and aspirated with wash buffer. The strips were then washed three times with PBS/0.05% Tween 20, allowing five minutes for each rinse.

The strips were then reacted with the staining reagents, which permit visualization of specific antibody binding to gp 120. The reagent chosen was horseradish-peroxidase. This reagent exhibits color when contacted by a working substrate which consists of 10 ml of PBS, pH 7.4, 2.0 ml of substrate stock, and 4.0 microliters of 30%  $H_2O_2$ . Substrate stock is made by dissolving 0.3 g of 4-chloro-1-naphthol in 100 ml of anhydrous methanol.

2.0 ml of Blotto/4% goat serum, containing 1:100 biotinylated goat anti-mouse IgG, was then added to each strip well. The trays were incubated at room temperature for thirty minutes on a rocking platform. The goat anti-mouse IgG conjugate will, of course, bind to any monoclonal antibody which has bound to the gp 120 on a strip.

The strip wells were then rinsed once with PBS/0.05% Tween 20, and shaken by hand several times to remove excess goat anti-mouse IgG conjugate. The wash buffer was discarded. The strip wells were then washed three times with PBS/0.05% Tween 20. Each washing lasted for five minutes.

2.0 ml of Blotto/4% goat serum containing 1:1000 horseradish-peroxidase-avidin D conjugate was added to each strip well. The avidin in this conjugate binds to the biotin in the goat anti-mouse IgG conjugate. Therefore the horseradish-peroxidase

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marker becomes linked to goat anti-mouse IgG and thereby marks any bound antibody. Following addition of the conjugate, the trays were incubated for thirty minutes at room temperature on a rocking platform.

Each strip well was washed three times with PBS/0.05% Tween 20, five minutes per wash, then once with PBS. 2.0 ml of the working enzyme substrate was added to each well, and the trays were incubated at room temperature until color developed. The working substrate solution contained 0.05% 4-chloro-1-naphthol and 0.01%  $H_2O_2$  in phosphate buffer saline at pH 7.4.

(iii) Results

As discussed above, the Western blot analysis was performed using antibody from the thirty-nine ELISA positive wells. With Western blot analysis only antibody from six of these thirty-nine wells was found to react with gp 120. All six of these wells were among the seven wells which had been found immunofluorescence positive in the immunofluorescence assay. Thus, only one of the seven immunofluorescence positive clones was not also positive in Western blot analysis.

h) Production and Purification of Monoclonal Antibodies

To produce large quantities of desired monoclonal antibodies, the following procedure was performed.

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The seven immunofluorescence positive clones, which have situated in the wells in the second twenty-four well plate, were grown up in a 100 mm tissue culture plate. The expanded culture of the selected seven single-cell clones were then separately injected into the peritoneal cavity of pristane treated mice, using five million cells per mouse. After seven days the ascites fluid of each mouse was collected and frozen.

The monoclonal antibodies in the ascites fluid were purified as follows. The frozen ascites fluid was thawed and filtered through a nylon cloth to remove viscous material. Sufficient phenylmethyl sulfonyl fluoride was added to the ascite fluid so that there was a final concentration of 0.1 mM. 0.05 ml of 1.2M acetate buffer (pH 4.0) was added for every milliliter of ascites fluid. The final concentration of the acetate buffer was 60 mM. The pH was adjusted to 4.5.

For every milliliter of treated ascites fluid, 25  $\mu$ l of caprylic acid (MW of 144.21, density of 0.91) was added dropwise with vigorous stirring. The suspension was kept at room temperature and stirred continuously for 30 minutes more.

The suspension was then centrifuged at 15,000 g for ten minutes in order to remove the precipitate. The supernatant, which contains IgG, was neutralized by adding a volume of 1 M HEPES buffer (pH 8.0) equal to one-tenth the volume of the supernatant. The IgG was then precipitated with 50%  $(\text{NH}_4)_2\text{SO}_4$ .

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The precipitate was then dissolved in HEPES saline buffer. This solution was dialysed overnight against HEPES saline buffer in order to remove  $(\text{NH}_4)_2\text{SO}_4$  from the IgG. The HEPES saline buffer was changed twice during the dialysis. After dialysis, the HEPES buffer saline contains purified dissolved IgG. The purified IgG was used in the infectivity assays and the syncytium formation assays which follow.

Example II: Verifying the Efficacy of the Invention

a) Neutralization Assay

An assay was performed to determine the effectiveness of the monoclonal antibodies of the invention in inhibiting infection of T-cells by HIV-1 virion. A comparison was made of the number of cells infected when HIV-1 alone was added to a cell culture, with the number infected when HIV-1 and the monoclonal antibodies of the invention were added. The cells selected for the neutralization assay were the H9 clones of the HT cell line.

i) Preparing the Virus, Antibody and Cells

H9 cells were prepared by washing a cell culture with H9 growth medium. The H9 growth medium contained 20% FBS (heat inactivated) in RPMI 1640, 5 mM of L-glutamine, 50 units/ml of penicillin, 50 mg/ml of streptomycin, and 5 mM of HEPES. The cells were then resuspended to a final concentration of  $2 \times 10^6$  cells/ml. The suspension was then incubated



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with 2 micrograms/ml of polybrene in a water bath at 37° for twenty minutes.

After incubation, the cells were spun down at 700 g for seven minutes. The supernatant was then discarded, and the cells were resuspended in H9 growth medium and washed again to remove the polybrene. The cells were then resuspended to  $2 \times 10^6$  cells/ml in growth medium.

Six of the seven immunofluorescence positive clones were chosen for use in the neutralization assay. The antibodies from the purified ascites (as described above) were sterilized by passing them through a 0.22 micron Millipore filter. The solution was then diluted in the H9 growth medium to yield different final concentrations of 100, 10, 1, 0.1, and 0.01 micrograms/ml.

Virus at 20 TCID<sub>50</sub>, or twenty times the TCID<sub>50</sub> value, was used in the infection of H9 cells. The TCID<sub>50</sub> value of the virus preparation was determined in previous infectivity assays under the same experimental conditions. It is defined as the virus titer at which 50% of the experimental wells are infected. 20 TCID<sub>50</sub> was equivalent to roughly a  $4.72 \times 10^{-5}$  dilution of the viral stock.

In the infectivity assays, 30  $\mu$ l of virus suspension, and 30  $\mu$ l of each of the antibody solutions, were mixed in the wells of a microtiter plate at 4° C for one hour. Each well was done in duplicate. The plate was then warmed in an incubator at 37° C and 5% CO<sub>2</sub> for thirty minutes.

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30  $\mu$ l of the polybrene treated H9 cell suspensions was then added to each well.

The microtiter plates were then incubated for one hour at 37° C in an incubator. 110  $\mu$ l of the growth medium was added to each well, bringing the total volume to 200  $\mu$ l. The plates were incubated for three days, and new growth medium was replaced every three days. Cells were collected on the third, sixth, ninth and thirteenth day.

The identical procedure described above was also performed using murine monoclonal antibody to human chorionic gonadotropin (anti-HcG) rather than one of the anti-HIV-1 antibodies of the invention. The cells treated with the anti-HCG antibody served as a negative control.

ii) Immunofluorescence Assay of Infected Cells

100  $\mu$ l aliquots of the cell suspensions collected on days 9 and 13 were washed with 3 ml of PBS. The cell suspension was centrifuged at 700 g for seven minutes and was washed again in PBS. The cells were finally resuspended in 50  $\mu$ l of PBS and 10  $\mu$ l of suspension was dotted onto a glass slide. This suspensions were air dried and then fixed with 1:1 acetone/methanol for ten minutes, air dried and stored at -20°C before assay.

In the assay, the fixed cells were rehydrated in PBS for twenty minutes and then incubated with 5% normal goat serum in PBS for another thirty minutes. After dripping away the excess normal goat serum, the cells were incubated at room temperature for one

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hour with anti-p24 monoclonal antibody (at a dilution of 1:100) containing 2% normal goat serum. This antibody binds specifically to the p24 core protein of HIV-1. The slides were kept in the humidifier to avoid drying. After the incubation, the slides were rinsed for three times in PBS for a total of 30 minutes. Then fluorescein conjugated goat anti-mouse IgG (F(ab')<sub>2</sub>) fragment was added at a dilution of 1:20. The slides were incubated for one hour at room temperature. The slides were then rinsed in three changes of PBS for thirty minutes and counterstained with 0.5% Evans blue for five minutes, washed and mounted in Fluoromount G. The cells were then observed under a fluorescence microscope.

The number of infected cells were counted at the magnification of 400x. Four data points were collected from each slide by random sampling over the field.

### iii) Results

The results are depicted graphically in Figures 1 and 2, where the percentage of immunofluorescence cells is plotted against the concentration of antibody in suspension. The results in Figure 1 are from cells collected on day 9. In Figure 2 the cells were collected on day 13.

Turning to Figures 1 and 2, it can be seen that four of the six antibodies tested (designated as BAT 123, 267, 509, and 085) were effective in inhibiting infection. In particular, BAT123 showed almost complete inhibition of infection on day 9. This

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results is to be contrasted with the negative control anti-HcG antibody, which exhibited virtually no inhibition. Nearly 100% of the cells treated with anti-HcG were immunofluorescent, irrespective of the concentration of antibody. The similar result was obtained with monoclonal antibody BAT 496 which is reactive with gp 120 but shows no neutralization activity. For this reason, BAT 496 was not assayed on day 13 and does not appear in Figure 2.

It should be noted that another antibody, BAT401, was tested for neutralization. However, the results do not appear in Figures 1 and 2 because it was found less effective in syncytium formation inhibition.

A comparison of Figures 1 and 2 shows that as time goes on, more of the cells in the suspension become infected. This result is expected. The amount of antibody in suspension available to neutralize the virus is decreasing due to change in medium and probably degradation or internalization. However, the infected H9 cells continually produce more virus, and this virus eventually infects all the cells.

The plots in Figures 1 and 2 show that with a decreasing concentration of antibody, a greater number of cells are infected. This indicates that the neutralizing effect of the antibodies is dosage dependent. The  $IC_{50}$  value of each monoclonal antibody, which is the dosage at which 50% of the cells are infected, was calculated. The results as taken on day 9 appear below in Table I.

TABLE I

<u>Monoclonal Antibodies</u>	<u>IC<sub>50</sub></u>
Anti-HcG (Negative Control)	$1 \times 10^5$ ng/ml
BAT085	100 ng/ml
BAT123	10 ng/ml
BAT267	10 ng/ml
BAT509	30 ng/ml
BAT496	$1 \times 10^5$ ng/ml

It can be seen that the monoclonal antibodies which are most effective at inhibition (BAT 123, 267 and 509), do so in nanogram quantities. This indicates that these monoclonal antibodies may also be very effective in minute quantities for in vivo AIDS therapy. The use of such minute doses would be a significant advantage over known therapeutic agents.

b) Inhibition of Syncytium Formation

Another test for the monoclonal antibodies of the invention was to determine whether they inhibited syncytium formation. Inhibition of syncytium formation would enhance the therapeutic value of the antibodies, inasmuch as the majority of cell infection and cell death in vivo is believed to occur via syncytium.

The syncytium assay was based on the assumption that the exterior envelope protein of the virus in infected H9 cells binds to the CD4 antigen which is carried by T cells. In the assay, infected H9 cells

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are added to a well containing CD4 DNA transfected HeLa cells. HeLa cells are used because they adhere, in a monolayer, to the bottom of the well. These transfected HeLa cells express abundantly CD4 antigen on their cell surface. Thus, they have the ability to fuse with infected H9 cells. Therefore, if syncytium formation occurs, aggregates of HeLa and H9 cells will be bound to the well. These multi-nucleated giant cells can readily be observed and counted.

The protocol for the syncytium formation assay is set forth below.

(i) Procedure for Syncytium Formation Assay

HeLa T4 cells (which express the CD4 antigen on the surface) were grown in a HeLa-T4 growth medium, which contained 5% FBS (heat inactivated) in DMEM, 5mM L-glutamine, 50 units/ml of penicillin, 50 mg/ml of streptomycin, and 5 mM of HEPES. The cells were harvested by trypsinization, to remove the cells from the flask, and washed. The cells were then seeded onto a 96 wells microtiter plate at a density of 10,000 cells per well. The plates were incubated at 37°C for thirty-six hours until 90% confluency was reached.

Both infected and uninfected H9 cells were then prepared. For preparing these cells, the cell suspension was first washed twice with H9 growth medium (20% FBS in RPMI 1640, 5 mM of L-glutamine, 50 units/ml of penicillin, 50 mg/ml of streptomycin

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and 5 mM of HEPES.) The cells were then resuspended in HeLa-T<sub>4</sub> at a concentration of 0.4 million/ml.

The antibodies were prepared by first performing a sterile filtration on the seven antibody solutions which had been used in the neutralization assay. Six of these solutions contained antibodies of the invention, and the seventh contained the anti-HcG. Each solution was then diluted to make two final concentration of 1.0 and 10  $\mu$ g/ml.

50 microliters of each antibody solution and 50 microliters of infected H9 cell suspension was added to the various wells of the microtiter plate. The microtiter plate wells had previously been coated with the HeLa T4 cells. In another HeLa T4-coated well, infected H9 cell suspension was added without the addition of antibody. This well was to serve as a positive control. In yet another coated well, uninfected H9 cell suspension was added. This well was to serve as a negative control. The experiments were done in triplicate.

The plates were then incubated for eighteen hours at 37° C and 5% CO<sub>2</sub>. The plates were washed gently twice with DMEM in order to remove unattached H9 cells. The DMEM was removed and the cells were fixed by adding 200  $\mu$ l of methanol per well for seven minutes. After removing the methanol, the cells were air dried, and then stained with 100  $\mu$ l of 1.4% methylene blue for ten minutes. The cells were rinsed with distilled water three times.

After staining, the cells were then observed under an inverted microscope (at a magnification of

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100 times), and the number of syncytia per field was determined. Aggregates of cells were considered to be a syncytium if more than five nuclei were present. Each well was counted three times randomly.

(ii) Results

The negative control well showed no syncytium formation. The results for the remainder of the wells appear below in Table II, expressed as a mean  $\pm$  standard deviation.



TABLE II

Inhibition of Syncytium Formation Between  
HIV-infected H9 Cells and HeLa T<sub>4</sub> Cells

<u>Antibody*</u>		<u>Number of Syncytia per Field</u>	<u>% Inhibition</u>
<u>&amp; Concentration</u>			
None		54.8 ± 3.6	0
Anti-HcG	1	50.0 ± 5.1	8.7**
	10	54.7 ± 7.6	0
BAT085	1	39.7 ± 2.8	27.6
	10	41.3 ± 6	24.6
BAT123	1	30.3 ± 4.5	44.7
	10	15.3 ± 4.7	72.0
BAT267	1	41.0 ± 6.6	25.2
	10	27.3 ± 5.7	50.2
BAT509	1	41.7 ± 4.9	23.9
	10	28.3 ± 3.3	48.5
BAT496	1	56.3 ± 9	0
	10	52.0 ± 3.6	5.1**

\* The 1.0 microgram/ml and the 10 microgram/ml solutions of antibody are designated "1" and "10" respectively.

\*\* Not significantly different from negative control.

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It can be seen from Table II that these results suggest that screening by the above-described methods is essential to identify the best antibodies for therapeutic use. The same antibodies which lowered infectivity of free HIV-1 virions (as shown in Figures 1 and 2) also were effective in inhibiting syncytium formation. BAT 123, 267 and 509 were particularly effective in both applications. BAT 496 was almost ineffective in both applications as was, of course, the negative control anti-HcG. Although BAT 085 was effective in neutralization, it was not among the most effective in syncytium inhibition.

BAT401 was not very effective at syncytium inhibition, although it was effective in the neutralization assay. This result indicates that antibodies which are effective in inhibiting HIV-1 infection are not necessarily effective in inhibiting syncytia formation. Accordingly, the three monoclonal antibodies of invention which were most effective (BAT123, 264 and 509) at inhibiting both infectivity by the HIV-1 virions and syncytium formation, were deposited at the American Type Culture Collection in Rockville, Maryland. They are available for inspection by the Patent and Trademark Office during the pendency of this application.

The Table II results demonstrate that, similar to neutralization as shown in Table I, syncytium inhibition is also dosage-dependent. The solutions with 10 microgram/ml of antibody were generally more

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effective in inhibition than the 1 microgram/ml solutions.

Example III: Neutralization of Different Strains and Isolates of HIV-1

Several antibodies were found to inhibit the infectivity of free HIV-1 virions and the syncytium formation between HeLa-CD4+ cells and H9 cells infected by HIV-1B. Since genomic analyses indicate that the virus mutates significantly both in vivo and in vitro (Alizon, M., Wain-Hobson, S., Montagnier, L. and Sonigo, P. (1986) Cell 46:63-74; Starcich, B.R., Hahn, B.H., Shaw, G.M., McNeely, P.D., Modrow, S., Wolf, H., Parks, E.S., Parks, W.P., Josephs, S.F., Gallo, R.C. and Wong-Staal, F. (1986) Cell 45:637-648), the application of these neutralizing monoclonal antibodies as agents for therapy and protection relies heavily on whether they are group-specific and protect HIV-1 infection caused by a large proportion of strains of the virus in the population. It is important to know whether BAT 123 and the other neutralizing monoclonal antibodies we raised recognize one or more distinct neutralization epitopes in the viral envelope protein gp120 with conserved amino acid sequences among different strains of HIV-1. In order to understand these characteristics of the antibodies, we studied whether these antibodies can inhibit the syncytium formation by other strains of HIV-1 with a substantial degree of heterogeneity in the amino acid sequence of gp120 (RF, AL, MN, Z84 and Z34) (Starcich et al., supra.). The neutralization

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antibody BAT 123 was chosen in the study because it was shown to elicit highest potency in the neutralization of the virus. In order to evaluate the effectiveness of the neutralizing antibodies on different HIV-1 variants existing in the infected population, we collected blood specimen randomly from infected individuals (in Houston, Texas; in Los Angeles, California; and in Boston, Massachusetts) with different disease states, and examined the effect of BAT 123 on the viral infection in the lymphocyte preparations by co-culture experiments.

a) Syncytium formation assay

Syncytium formation assay was performed as described in Example 2.

b) Co-culture assay

The procedure used is similar to that described earlier, 30 ml of heparinized blood from each patient was freshly collected and processed for mononuclear leukocytes by density-gradient centrifugation. Briefly, the whole blood was diluted with equal volume of phosphate-buffered saline (PBS). 25 ml of the diluted blood was laid over 10 ml of Ficoll-Paque (Pharmacia) and centrifuged at  $1500 \times g$  for 30 minutes; at the end of the centrifugation, the interphase containing mononuclear leukocytes was removed and washed twice in PBS. The mononuclear leukocytes were then cultured at  $0.5 - 1 \times 10^6$ /ml in the RPMI 1640 medium supplemented with

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15% heat-activated fetal bovine serum, 2mM L-glutamine, 10% interleukin-2 (Cellular Products), 25 neutralizing units/ml sheep anti-human alpha interferon (Interferon Science), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 2  $\mu$ g/ml Polybrene. Equal volume of phytohaemagglutinin (PHA)-stimulated mononuclear leukocytes from normal donor blood was mixed with the patient culture. The mononuclear leukocytes from the normal donor blood was stimulated for one day early with 2  $\mu$ g/ml PHA-P (Sigma). They were washed twice in PBS to remove the lectin. BAT 123 was added to the test culture at the final concentration of 10  $\mu$ g/ml. The total volume of the culture was 10 ml. Five ml of the cell culture was removed at 3 - 4 day intervals, centrifuged at 1,500 x g for 15 minutes to remove the cells and debris. The supernatants were collected and assayed for reverse transcriptase activities after precipitation of the virus using 10% polyethylene glycol (PEG) (Gupta, P., Galachandran, R., Grovit, K., Webster, D. and Rinaldi, C. Jr. (1987) J. Clin. Microbiology 25:1122-1125).

c) Reverse transcriptase assay

The procedure for the measurement of reverse transcriptase activity was described earlier (Barre-Sinoussi, F., Chermann, J.C., Rey, F. Nugeyre, M.T., Charmaret, S., Gruest, J., Daugnet, C. Axler-Blin, C., Vezinet-Brun, F., Ronziou, C., (1984) Science 220:86-87). Briefly, the PEG-precipitated virus was solubilized for 20 minutes in 100  $\mu$ l of Tris-

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buffered saline (pH 8.2) containing 0.1% Triton X-100, 2mM dithiothreitol, 0.2mM leupeptin and 50 mM -amino-n-caproic acid. In the assay, 100  $\mu$ l of the substrate solution in 50 mM Tris-HCl pH 8.2 containing 8mM  $MgCl_2$ , 20  $\mu$ Ci  $^3H$ -thymidine triphosphate (2mCi/ml), 0.05 units of template-primer poly(rA). poly(dT)<sub>12-18</sub> was added to 25  $\mu$ l of the solubilized virus. No template-primer was added to the corresponding control, but substituted with distilled water instead. The reaction mixtures were incubated at 37°C for one hour and the reaction was terminated by addition of 5% cold trichloroacetic acid and finally filtered over Whatman GF/C filters which were washed thoroughly and counted for radioactivity using a scintillation counter. The specific reverse transcriptase activities were calculated as the difference in radioactivity when the template-primer was added.

### Results & Discussion

We studied the neutralizing monoclonal antibodies claimed with regard to their group-specificity to the virus and their cross-protection to six different HIV-1 strains (HIV-1<sub>B</sub>, HIV-1<sub>RF</sub>, HIV-1<sub>AL</sub>, HIV-1<sub>MN</sub>, HIV-1<sub>Z84</sub>, and HIV-1<sub>Z34</sub>). In syncytium formation assay between HeLa-CD4+ cells and H9 cells chronically infected with these strains of HIV-1 respectively, BAT 123 at 25  $\mu$ g/ml inhibited syncytium formation by almost 80%. It also reduced the syncytium formation of H9 cells infected with

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HIV-1<sub>MN</sub>, HIV-1<sub>AL</sub>, HIV-1<sub>RF</sub> and HIV-1<sub>Z34</sub> by approximately 50%, and HIV-1<sub>Z84</sub> by 23%. (See Table III).

TABLE III

CROSS-PROTECTION OF SYNCYTIUM FORMATION BY  
H9 CELLS INFECTED WITH DIFFERENT HIV-1 STRAINS

<u>Infected H9</u> <u>Cells</u> <u>Inhibition</u>	<u>With</u> <u>Antibody</u>	<u>Without</u> <u>Antibody</u>	<u>% of</u>
H9 uninfected (control)	---	---	---
H9 - HIV - 1 <sub>B</sub>	2.33 ± 0.51*	10.25 ± 0.99	77.3
- HIV - 1 <sub>MN</sub>	2.08 ± 0.38	4.25 ± 0.46	51.0
- HIV - 1 <sub>AL</sub>	7.08 ± 0.66	13.91 ± 1.27	49.1
- HIV - 1 <sub>RF</sub>	1.91 ± 0.55	3.91 ± 0.47	51.0
- HIV - 1 <sub>Z84</sub>	12.41 ± 1.46	16.08 ± 0.55	22.8
- HIV - 1 <sub>Z34</sub>	1.58 ± 0.14	3.08 ± 0.55	48.7

\* Expressed as number of syncytia per microscopical field ( $\bar{x} \pm S.E.$ ,  $n = 11$  or  $12$ ),  $p < 0.05$ , paired student's  $t$  test.

In the co-culture experiments using lymphocytes isolated from the peripheral blood of patient clinically diagnosed positive asymptomatic state, AIDS or ARC; out of 32 patient blood specimen tested, the virus had been isolated from 18 samples as measured for reverse transcriptase activities. When 10  $\mu$ g/ml BAT 123 was added in the culture

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medium throughout the experiments,--the viral replication was inhibited in all of the 18 virus-positive cultures. The degree of inhibition ranged from 43.7 to 100%. Among the 18 samples, 8 samples were effectively inhibited by over than 90%. (See Table IV).

The results from our in vitro experiments suggest that the neutralizing monoclonal antibody BAT 123 is group-specific and can cross-protect different diverse strains of HIV-1 in the syncytium formation assays and inhibit viral infection in patient blood specimen.

CO-CULTURE EXPERIMENTS  
Reverse Transcriptase Activity (cpm)

Patient No.	Control	With BAT 123	Percent Inhibition	T4 Cell Count/ul	Total Lymphocyte/ul	Clinical States
7	720123	335156	53.4	180	2250	+, asym
8	N.D. *			398	1443	ARC
9	N.D.			180	2178	+, asym
10	100825	38283	62.0	0	5044	AIDS (PCP)
11	331689	186660	43.7	110	2210	AIDS (PCP)
12	317107	104	99.9	14	462	AIDS (PCP,KS)
13	N.D.			261	1440	ARC
14	N.D.			23	2310	AIDS (PCP)
15	9081	0	100.0	229	1590	AIDS (PCP)
16	66224	14382	72.4	29	400	AIDS (PCP)
17	65991	5593	91.5	25	2553	ARC
18	N.D.			715	3502	ARC
19	N.D.			825	2886	+, asym
20	N.D.			948	2964	ARC
21	N.D.			151	1892	ARC
22	22034	829	96.2	171	2444	AIDS (PCP)
23	76103	1004	98.6	140	870	AIDS (PCP)
24	N.D.			503	2400	+, asym
25	166167	10900	93.4	163	3264	AIDS (PCP)
26	171670	66576	61.2	74	530	+, asym
27	293485	143301	51.1	8	2016	AIDS
28	16884	146	99.1	33	1050	AIDS
29	38703	9104	76.0	197	1364	AIDS
30	20863	1298	93.8	178	3570	AIDS
31	N.D.			168	2808	AIDS
32	284570	102787	63.9	265	3716	AIDS
33	N.D.			30	594	AIDS
34	Blood not processed**			33	1664	AIDS
35	N.D.			721	4200	+, asym
36	N.D.			723	2784	+, asym
37	43108	14062	67.4	42	4355	+, asym
38	N.D.			516	3036	+, asym
39	50256	8019	84.0	10	350	AIDS

\* N.D. = Not detected

\*\* Specimen from VA-34 was not processed since there was not enough blood.

asym = asymptomatic

AIDS = acquired immunodeficiency syndrome

ARC = AIDS related complex

PCP = Pneumocystic carinii pneumonia

KS = Kaposi's sarcoma

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Example IV: Determining The Peptidic Segments Of  
Gpl20 Reactive With Monoclonal Antibodies

Methods

In order to map the epitopes on gp120 of HIV-1 that are recognized by the monoclonal antibodies, we have determined using Western blot assays the reactivities of some of the monoclonal strips. The strips were obtained from Dr. Steve Petteway, Medical Products Department, DuPont de Nemours and Company, Wilmington, Delaware. The synthetic peptides on the strips are 8-20 amino acid residue long. These peptides represent overlapping peptidic segments across the entire length of gp120 of HIV-1B strain. Several tens of peptide solutions had been adsorbed on individual strips in equally spaced regions and the strips were provided to us in a dry form.

The immunoblotting procedure using the nitro-cellulose strips is the same as the Western blot procedure used to determine whether the monoclonal antibodies react with gp120 described in the preceding section.

Results

Three of the monoclonal antibodies BAT123, BAT267, and BAT085 showed very clear and specific reactivities with particular peptides in the Western blot assay.

BAT267	PPNNNTRKSIRIQRG (residue #244-308)
BAT123	RIQRGPGRAFVTIGK (residue #304-318)
BAT083	VQKEYAFFYKLDIIP (residue #174-188)

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The 15 amino acid long peptides reactive with BAT267 and BAT123 overlap by 5 amino acids. However, the antibodies react with just one of them and do not react with the other to any measurable extent. The antibodies do not react with peptides overlapping at the other ends either, i.e. BAT267 does not react with LNQSVRINCTRPNNN and BAT123 does not react with VTIGKIGNMRQAHCN. These results suggest that the antibodies react with an epitope borne by either all or a part of the middle five amino acids or a combination of these amino acids with some of the flanking amino acids. Similar findings have been made for BAT085 and similar conclusions may be made for it.

Example V: Cloning and Identification of the Functionally Rearranged V<sub>L</sub> and V<sub>H</sub> Genes of BAT123

The cloning of the functionally rearranged V<sub>L</sub> and V<sub>H</sub> genes of BAT123 was accomplished by the screening of BAT123 genomic libraries using appropriate molecular probes in a strategy similar to that described by Oi and Morrison (Biotechniques, 4:214-221). The identification and final verification of the cloned gene segments was achieved by the aid of the nucleotide sequences of mRNA's for BAT123 immunoglobulin molecules. The rationale is based on the fact that only when a variable region gene segment is appropriately joined to the J region

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gene in the case of  $\kappa$  chain rearrangement or appropriate VDJ joining occurs in the case of heavy chain rearrangement, is the full length and properly spliced immunoglobulin mRNA synthesized in the antibody producing cells.

The sequences of these mRNA's, determined either by direct sequencing of the mRNA molecule or from the cDNA clones are therefore most suitable to serve as a guide for the selection and verification of the functionally rearranged variable region genes. Any gene segment containing sequences identical to the RNA sequence can be considered functionally rearranged.

The sequences of the mRNA molecules corresponding to the variable regions were determined by a primer extension/dideoxynucleotide termination method with the use of mRNA prepared from the polysomes of the BAT123 hybridoma cells. This direct RNA sequencing approach eliminates the intermediate cDNA cloning step in the conventional approach to derive the mRNA sequence; hence, it provides a relatively fast way to determine the RNA sequence.

The primers used for this RNA sequencing were 5'dTGGATGGTGGGAAGATG3' for light chain mRNA and 5'dGGCCAGTGGATAGAC3' for heavy chain mRNA (both primers were obtained from Pharmacia, Nutley, NJ). These oligonucleotides were complementary to the mRNA sequences in the constant regions at positions proximal to the junctions of J and C regions of the molecules. Primer extension was accomplished with

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the use of AMV reverse transcriptase and terminated by dideoxynucleotides. The nucleotide sequence of mRNA's was determined by the gel electrophoresis and subsequent autoradiography.

A genomic DNA library for BAT123 cells was constructed in lambda phage vector  $\lambda$ 2001 (Karin, J., Natthes, W.D.H., Gait, M.J., Brenner, S. (1984) Gene 32:217-274). High molecular weight genomic DNA from BAT123 hybridoma cells was partially digested with restriction endonuclease Sau 3AI and size fractionated on a 10-40% sucrose density gradient. DNA fragments of 18-23 kilobase pairs (Kbp) were ligated with  $\lambda$ 2001/BamH1 arms (Stratagene Cloning Systems, La Jolla, CA). and packaged by using Gigapack Gold packaging extracts (Stratagene). This genomic library was first screened for the functionally rearranged variable region gene of BAT123 light chain ( $V_L$ ). The probes used for this screening included a 2.7 Kbp Hind III DNA fragment containing all of the mouse germline kappa ( $\kappa$ ) chain joining regions J1-J5 ( $J_\kappa$  probe; Max, E.E., Maizel, J.V., and Leder, P. (1981) J. Biol. Chem. 256:5116-5120) and two oligonucleotide probes  $V_\kappa$ -1 and  $V_\kappa$ -2 derived from the nucleotide sequence of BAT123 light chain mRNA. The sequences of these oligonucleotide probes are  $V_\kappa$ -1: 5'dTTTGCTGACAGTAATAGG3' and  $V_\kappa$ -2: 5'dATATAACTATCACCATCA3'. The probes were synthesized by using the phosphoramidite chemistry on an Applied Biosystems DNA synthesizer model 381.

Approximately  $5 \times 10^5$  phage recombinants were screened initially with  $^{32}$ P-labeled mouse  $J_\kappa$  DNA

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probe. Plaque hybridizations were carried out in 5xSSC with 50% (v/v) formamide at 42°C for 16 hours (1xSSC=0.15M NaCl, 0.015M sodium citrate). Final washes were in 0.2x SSC/0.1% SDS at 65°C. Two positive clones were obtained. They were subsequently screened with the use of <sup>32</sup>P-labeled oligonucleotide probes V<sub>κ</sub>-1 and V<sub>κ</sub>-2. Hybridization with the oligo-probes was carried out in 5x SSC at 37°C for 18 hours and washes were carried out in 2x SSC/0.1% SDS at room temperature. One of these clones, V<sub>κ</sub>123-23 was shown to hybridize with the J<sub>κ</sub> DNA probe and both oligonucleotide probes. DNA sequence determination of this clone by the dideoxynucleotide termination method showed that it carried a V<sub>L</sub> gene segment with sequence identical to that determined from BAT123 light chain mRNA. This clone was used in the subsequent construction of the mouse/human chimeric L chain gene.

For the cloning of the functionally rearranged variable region genes for BAT123 heavy chain (V<sub>H</sub>), partial genomic libraries were prepared. Genomic Southern blots of the EcoRI digest with the J<sub>H</sub> probe (see below) had previously revealed 2 potentially functionally rearranged V<sub>H</sub> genes in BAT123, one being 7.5 Kbp and the other 4.5 Kbp, in addition to the 6.6 Kbp fragment which was presumably derived from the fusion parent of BAT123, i.e. NS-1 cells. Two partial libraries containing these DNA bands were prepared. High molecular weight DNA was digested with EcoRI to completion and fractionated on a 0.7% agarose gel. DNA fragments of the size

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4-6 Kbp and 6-9 Kbp were isolated and ligated with lambda vector  $\lambda$ gtWES $\lambda$ B (Leder, P., Timeier, D., and Enquist, L. (1977) Science 196:175-177). The ligated DNAs were packaged and recombinant plaques were screened. The probes used included a 2 Kbp BamHI-EcoRI DNA fragment containing the mouse H chain joining regions J3 and J4 ( $J_H$  probe; Gough, N.M. and Bernard, O. (1981) Proc. Natl. Acad. Sci. USA 78:509-513) and an oligonucleotide probe VH-1, 5'dAGTGTGGCTGTGTCCTC3' derived from BAT123 mRNA sequence. The hybridization conditions for these probes were as described above for  $\kappa$  chain. Screening of these two EcoRI partial libraries with  $J_H$  probe resulted in the isolation of 3 independent phage clones containing a 7.5 Kbp, 6.6 Kbp or a 4.5 Kbp DNA fragment. Subsequent hybridization using oligonucleotide probe VH-1 revealed that only the phage clone containing 4.5 Kbp insert, clone  $V_H$ 123-E3, hybridized with the probe. DNA sequencing of this clone showed that it contained a  $V_H$  sequence identical to that from  $V_H$  mRNA of BAT123. This clone was used in the construction of the mouse-human chimeric H chain gene.

#### Fusion of Murine V with Human C exons and Introduction into Murine Myeloma Cells

The functionally rearranged L and H chain V genes isolated from BAT123 cells were joined to human  $\kappa$  and  $\gamma$ 1 C region genes in expression vectors containing dominant selectable markers, neo

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(Southern, P.J. & Berg, P. (1981) J. Mol. Appl. Genet. 1:327-341) and gpt (Mulligan, R.C. and Berg, P. (1981) Proc. Natl. Acad. Sci. USA 78:2072-2076), respectively. To construct the desired chimeric gene, the Hind III fragment of pV184ΔHneo.DNSV<sub>L</sub>-hC<sub>κ</sub> (Oi, V.T. and Morrison, S.L. (1986) Biotechniques 4:214-221) containing the dansyl-specific V<sub>L</sub> gene was replaced with the 4.4 Kbp Hind III fragment containing the L chain gene of BAT123 derived from clone Vk123-23. The structure of the resulting plasmid pSV184ΔHneo.BAT123.VkhC<sub>κ</sub> is shown in Figure 1A.

The chimeric H chain gene was constructed by replacing the EcoRI fragment in the pSV2ΔHgpt.DNSV<sub>H</sub>.hC<sub>γ1</sub> plasmid containing the dansyl-specific V<sub>H</sub> gene with the 4.5-kbp EcoRI fragment containing the functionally rearranged BAT123 H chain gene derived from the phage clone VH123-E2. The structure of the resulting plasmid pSV2ΔHgpt.BAT123.V<sub>H</sub>-hC<sub>γ1</sub> is shown in Figure 1B.

The L and H chain chimeric genes shown in Figure 3 were used to transfect mouse myeloma cells. The myeloma cells chosen, Sp2/0, is a non-secretory cell line (Shulman, M., Wilde, C., and Kohler, G. (1978) Nature 276:269-270) that does not produce immunoglobulin molecules of its own. The calcium phosphate precipitation method (Graham and van der Eb (1973) Virology 52:456) was adopted to transfer the chimeric genes into Sp2/0 cells.

To facilitate the DNA transfection Sp2/0 cells were seeded at  $5 \times 10^6$  cells per 100-mm Petri dish

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which had been previously treated with histone (Sigma Chemical Co., St. Louis, Mo) and incubated for 16 hours at 37°C. Approximately  $7.5 \times 10^7$  Sp2/0 cells were cotransfected with CsCl-ethidium bromide gradient purified pSV184 $\Delta$ Hneo.BAT123-VkhC $_{\kappa}$  (150  $\mu$ g) and pSV2 $\Delta$ Hgpt.BAT123.V $_{H}$ .hC $_{\gamma 1}$  (150  $\mu$ g) using calcium phosphate precipitation method. Two days after transfection, cells were subcultured in microtiter plates at a density of  $1 \times 10^5$  cells per well in culture medium containing 400  $\mu$ g/ml G418 and 0.2  $\mu$ g/ml mycophenolic acid. The frequency of transfectants resistant to both selection drugs was approximately  $2 \times 10^{-5}$ .

The stable transfectants (transfectoma) were screened for the production of secreted, functional chimeric antibodies by virtue of their affinity for purified HIV gp120, a distinct characteristic of BAT123. Purified gp120 was immobilized on microtiter plates, and allowed to react with culture supernatants from the transfectants. The antigen-antibody complexes were detected with alkaline phosphatase-conjugated antisera specific for human IgG. As shown in Table V, 707 of the 1200 transfectants tested gave a positive signal in the ELISA, indicating that the intact chimeric antibody was secreted, and that this protein retained the ability to bind HIV gp120. The untransfected parental cell line gave a negative result.



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TABLE V Level of Secretion of Chimeric Antibody by Transfectomas

Recipient Cell	Number Transfectoma Tested	Number Negative (-)	# Weakly Positive (+)	Number Positive (+)	Per Cent Positive
SP2/0	1200	493	511	196	59

Transfectomas were scored based on their OD in ELISA. "Negative" is defined as OD of 0.0 to 0.1, "Weakly positive" denotes 0.1 to 0.2 and "positive" indicates 0.2-3.0.

#### Production of Chimeric Antibody from Transfectoma cell Lines

Seventeen transfectoma lines exhibiting OD greater than 1.0 in ELISA were selected and the chimeric antibody producing cells were purified by single cell cloning technique from which twelve stable cell lines were established. These transfectoma cell lines were then tested for stability of chimeric antibody production in the absence of selection drugs G418 and mycophenolic acid. The cells were cultured in the medium with stepwise reduction in the two selection drugs at 2-week intervals which resulted in the complete elimination of the drugs. During each reduction of drugs the production of the chimeric antibody in these cell lines were monitored by ELISA. Three of these cell lines lost their ability to secrete chimeric antibody upon removal of selection pressure. The

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remaining 9 cells lines remain stable in the production of chimeric antibody at 5 weeks after complete elimination of selection drugs in the culture medium.

To estimate the level of chimeric antibody production and to prepare the antibody for further characterization, transfectoma line CAG1-51-4 was expanded and grown in tissue culture medium. Approximately 600 ml of culture medium was collected and from which 14.4 mg (estimated by BCA protein assay, Pierce, Rockford, IL) of the chimeric antibody was purified by utilizing r-protein A-Sepharose affinity column (Repligen Corporation, Cambridge, MA). The IgG concentration in the culture supernatant of this transfectoma cell line was therefore estimated to be 24  $\mu\text{g/ml}$ , a level somewhat higher than the level produced by BAT123 hybridoma cells (20  $\mu\text{g/ml}$ ).

#### Biochemical Analysis of Chimeric Antibody

Purified chimeric immunoglobulin was used to characterize the biochemical/immunological properties of the chimeric antibody.

##### A) Isoelectric points

The pattern of isoelectric focusing (IEF) gel of the purified chimeric antibody along with that of BAT123 is shown in Figure 4. The IEF pattern was

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obtained by application of purified antibody samples onto Pharmacia's Phast System<sup>TM</sup> and IEF was carried out according to the procedure recommended by the manufacturer. The IEF pattern indicated that the chimeric antibody contained two major species of molecules with pI in the range of pH 6.8 - 7.2 whereas the corresponding molecules of BAT123 exhibited pI in the range of pH 5.6 to 5.8. The replacement of constant regions of the immunoglobulin molecule from mouse to human therefore greatly altered the composition of the antibody which is reflected in the IEF pattern.

B) Reactivity of the Chimeric Antibody to anti-mouse as well as anti-human antisera

When the chimeric antibody was subjected to a 10% SDS-polyacrylamide gel electrophoresis under reducing conditions (Laemmli, U.K. (1970) Nature 227:680-685) two bands were observed (Figure 5A, Lane 2). Protein band of molecular weight of 53,000 daltons corresponds to the chimeric heavy chains, and exhibits a close similarity in size to the heavy chains of BAT123 immunoglobulin (Figure 5A, Lane 1). The protein band with the size of approximately 23,000 dalton denotes the light chains of antibodies. The slightly slower mobility observed for chimeric light chains is also seen in other chimeric antibody and may not necessarily be attributed to the larger size of human light chain  $\kappa$  constant region ( $hC_{\kappa}$ ) than the murine counterpart. The fully

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assembled  $H_2L_2$  molecule of the chimeric antibody shows identical mobility to that of BAT123 (mw 146,000 dalton) when the immunoglobulins were resolved in the 10% SDS-PAGE under non-reducing conditions (Figure 5A, Lanes 3 & 4).

To test whether the chimeric antibody indeed incorporated the constant regions of human immunoglobulin, 2  $\mu$ g of the chimeric antibody and BAT 123 were electroblotted onto a nitrocellulose membrane (100 volts, 1 hour in a transblot buffer consisting of 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3) after the proteins were resolved in 10% SDS-PAGE under reducing conditions in a BioRad Mini-Protein II Dual Slab Cell apparatus. One of the replica membrane filters was reacted with biotinylated anti-mouse antibody (Vector Laboratory, Burlingame, CA) whereas the other filter was reacted with biotinylated anti-human antibody in Blotto buffer consisting of 5% non-fat dry milk in phosphate buffered saline (PBS). After 1 hour incubation at 37°C, the membrane filters were washed with 2 changes of PBS + 0.1% Tween 20 (PBST) and both membranes were reacted with horseradish peroxidase-avidin conjugate in Blotto at room temperature for 30 minutes. After final washes with PBST the reactive protein bands were visualized by color development using 4-chloro-1-naphthol (4-CN) and hydrogen peroxide. As shown in Figure 5B, BAT123 immunoglobulin was extensively reactive with anti-mouse antiserum whereas chimeric antibody is only barely reactive to the same serum. On the other

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hand, chimeric antibody reacted strongly with anti-human antiserum whereas BAT123 did not show appreciable reactivity to the antiserum (Figure 5C). This result demonstrated that portions of the chimeric antibody molecule was indeed derived from human immunoglobulin.

### C) The IgG Subclass of the Chimeric Antibody

The heavy chain chimeric gene was constructed by splicing the  $V_H$  gene of BAT123 into the coding sequence of human  $C_{\gamma 1}$  region. The resulting chimeric antibody was therefore expected to be of IgG1 subclass. To confirm the isotype of the expressed constructed chimeric antibody the following experiment was conducted. Mouse anti-human IgG1 and IgG3 anti-sera (Fisher Biotech) were separately coated onto microtiter wells in series of 2-fold dilutions. Chimeric antibody at 20  $\mu\text{g/ml}$  was added to these wells and incubated at 37°C for 1 hour. After washes with PBST the complexes were detected by incubation with goat anti-human-horseradish peroxidase conjugate (Vector) with a subsequent color development. The result (shown in Figure 6) clearly demonstrated that chimeric antibody is of IgG1 subclass.

D) Antigen Specificity of the Chimeric Antibody

The amino acid residues within an immunoglobulin molecule that are directly involved in the formation of the antigen binding site are generally believed to be in the complementarity determining region (CDR) which reside in the variable domain (V) of the immunoglobulin. To determine if the chimeric antibody retained the antigen specificity of the parent antibody the following experiments were performed. In the first experiment the chimeric antibody was allowed to react with a commercially available immunoblot strip (gift of Dr. Robert Ting, Biotech Research Labs) that contains all antigens of purified HIV resolved in SDS-PAGE with a subsequent electrotransblot on to nitrocellulose membrane filters. The reaction was carried out at room temperature for 16 hours in blotting buffer. The reactive complexes were then detected by incubations first with biotinylated-anti-human antisera (Vector) followed by avidin-horseradish peroxidase with washes in between each incubation step and finally visualized by color development with the enzyme substrates 4-CN and hydrogen peroxide. The result was shown in Figure 7A. The antigen band that reacted with the chimeric antibody (lane 3) was identical to the one detected by BAT123 antibody in a parallel reaction (lane 2) and corresponded to the viral envelope glycoprotein gp120 in a viral antigen profile displayed by reaction with a reference serum from patient with AIDS. This result showed that the chimeric antibody retained the antigen specificity of BAT123 to bind HIV-gp120.

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To further test whether the chimeric antibody recognizes the same antigenic determinant (epitope) within gp120 as does BAT123, the antibody was allowed to react with a membrane strip containing a series of 32 overlapping oligopeptides that represent the potential antigenic determinants in gp120 (gift of S. Petteway, Du Pont). The incubation procedure was essentially the same as that for the immunoblot strip of viral antigens. The result (shown in Figure 7B) indicates that the chimeric antibody binds to the same oligopeptide as does BAT123 which has the amino acid sequence of Arg.Ile.Gln.Arg.Gly.Pro.Gly.Arg.Ala.Phe.Val.Thr.Ile.Gly.Lys

The antigen specificity of the murine antibody BAT123 was therefore well preserved upon conversion into a mouse/human chimeric antibody.

#### Biological Activity of the Chimeric Antibody

##### A) Neutralization of HIV-1 infection to H9 Cells by Chimeric Antibodies

The ability of the chimeric antibodies to neutralize the HIV-1 infection to H9 cells was measured using the similar procedure described earlier (U.S. patent application Serial No. 137,861 filed December 24, 1987, which is a continuation-in-part of U.S. Application Serial No. 057,445, filed May 29, 1987). The virus used was prepared from culture supernatants of HTLV-IIIB-infected H9 cells. 40 ml of cell-free supernatant was

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centrifuged at 35,000 x g for 3 hours. The pellet was resuspended in 3 ml of growth medium. The titer of the virus was measured by infecting H9 cells with the viral stock in ten-fold serial dilutions. The TCID<sub>50</sub> of the viral stock was determined as the infective dose at which half of the number of the minicultures was infected.

In the neutralization assay, an infectivity dose equivalent to 20 times of the TCID<sub>50</sub> was used. 50  $\mu$ l of 60 TCID<sub>50</sub> of the viral stock was pre-incubated with 50  $\mu$ l the antibodies tested in a microculture well of a 96-well plate. The antibodies tested were the chimeric antibody CAG1-51-4, the murine monoclonal antibody BAT123 and a murine monoclonal antibody to human chorionic gonadotropin (anti-hCG). In the control, 50  $\mu$ l of growth medium without any antibodies was used. The mixtures were kept at 37°C in a 5% CO<sub>2</sub> incubator for 1 hour. The final concentration of the antibody was 100, 50, 25, 12.5 and 6.25  $\mu$ g/ml. Each concentration of the tested antibodies was performed in triplicate. At the end of the incubation, 50  $\mu$ l of  $4 \times 10^6$ /ml H9 cells was added. The H9 cells were harvested in log phase and pre-incubated for 1 hour at 37°C with 2  $\mu$ g/ml polybrene in the RPMI-1640 growth medium containing 15% heat-inactivated fetal bovine serum before being added to the mixture. At the end of the incubation, the cells in each microculture wells were resuspended, 40  $\mu$ l of the cell suspension was added to 200  $\mu$ l of fresh growth medium in the corresponding wells of another microculture plates.



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The microculture plates were kept at 37°C in 5% CO<sub>2</sub> in an incubator. On day 3, day 5, day 7, day 9, day 11, and day 14 150 µl of cell suspension from each microculture was removed and placed into a U-bottomed well of another 96-well plate. The plate was centrifuged at 200xg for 5 minutes. The supernatants were collected for HIV-1 viral antigen capture assays. The wells were fed with 150µl fresh growth medium.

In the antigen capture assay, the HIV-1 specific antigens in the cell-free supernatant were measured by virtue of their affinity for the immunoglobulins from patients with AIDS. One hundred µl of diluted purified AIDS patient immunoglobulin (1:2000, approximately 5µg/ml) was added to each well of a Cobind plate and incubated for 2 hours at 37°C. Then the wells were rinsed two times with 200 µl of phosphate-buffered saline (PBS). The wells were blocked with 220 µl of 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Then it was rinsed three times with PBST (PBS containing 0.1% Tween 20). The wells were then emptied. 50 µl of the test samples (undiluted or in appropriate dilution) together with 50 µl of PBSTB (PBS containing 1% BSA and 0.1% Tween 20) were added to the well. The negative control contained 50 µl of the growth medium. The plate was incubated for 1 hour at 37°C. Then it was rinsed three times with PBST. 100µl of diluted peroxidase conjugated AIDS patients immunoglobulins was added to each well for 1 hour at room temperature. The plate was then rinsed three times

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with PBST. 100 $\mu$ l of a substrate solution (containing 20 mM sodium acetate buffer pH 6.0, 0.001% 3,3',5,5' tetramethylbenzidine and 0.001% hydrogen peroxide) was added to each well and incubated for 30 minutes at room temperature. Then 50 $\mu$ l of 2M H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction. The absorbance was read at 490 nm. The readings from the triplicate were averaged and compared between the control and the test antibody for the percent of inhibition when neutralizing antibodies were added. The results (Figure 8) showed that at all dilutions the chimeric antibody completely neutralized the HIV-1 infection to H9 cells at a 14-day assay. This neutralizing activity was identical to that of the parent murine antibody BAT123. A control murine antibody (anti-hCG) and the growth medium did not exhibit any inhibitory activity.

B) Inhibition of Syncytium Formation by Chimeric Antibody

The HIV-neutralizing activity of the chimeric antibody was also assessed by its ability to inhibit syncytium formation. The effects of chimeric antibody on HIV-1 transmission via cell fusion were studied using HIV-1 infected H9 cells and CD4-expressing HeLa cells (HeLa-CD4+), which fuse upon contact and form syncytia in culture. HeLa is a human carcinoma cell line. HeLa-CD<sub>4</sub>+ contains in its genome, CD4 encoding DNA introduced by

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transfection and thus, it expresses CD4 antigen on its cell surface. The HeLa-CD<sub>4</sub><sup>+</sup> cell line was a gift from David. D. Ho (University of California, Los Angeles). A culture of HeLa-CD<sub>4</sub><sup>+</sup> cells were plated onto wells of 96-well microculture plates at 20,000 cells per well. The plates were incubated for 36 hours and by this time, the monolayer epithelial cells were almost confluent. When  $1 \times 10^4$  infected H9 cells were added to the confluent HeLa-CD<sub>4</sub><sup>+</sup> cells, the cells formed contacts and fused, and by 18 hours multinucleated giant cells (syncytia) formed. Those syncytia with more than five nuclei could be easily identified and enumerated, thus providing quantitative measurements of syncytium formation. When the effects of chimeric antibodies on syncytium formation were studied, antibodies at different concentrations were mixed with infected H9 cells and added to the HeLa-CD<sub>4</sub><sup>+</sup> cells. The final total volume of the culture medium per well was 200  $\mu$ l. At the end of 18 hours incubation at 37°C, the wells were washed with PBS, fixed with methanol, air-dried, and stained with methylene blue. The cells were examined at 100X magnification and the number of syncytia (of more than 5 nuclei) were determined in four randomly chosen fields and averaged. As shown in Table VI, the chimeric antibody CAG1-51-4 gave 89.1%, 65.7%, and 58.6% reduction in the number of syncytia formed between H9 cells and HeLa-CD<sub>4</sub><sup>+</sup> cells at levels of 20  $\mu$ g/ml, 10  $\mu$ g/ml, and 5  $\mu$ g/ml, respectively. This effect is essentially identical to that caused by BAT123 and



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it indicates that the chimeric antibodies retained the activity to inhibit the fusion between the HIV-infected cells and uninfected cells, one of the major routes for HIV transmission

The control murine antibody anti-hCG gave no effect on the syncytium formation.

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Table VI Inhibition of Syncytium Formation Between  
HIV-1 Infected H9 Cells and HeLa-CD4+  
Cells by Chimeric Antibody

Antibody Tested	Number of Syncytia* per field	Per Cent Inhibition
Control (no antibody)	37±2.6**	
BAT123 20 µg/ml	3.67±2.1	90.0
10 µg/ml	11.3±2.1	69.5
5 µg/ml	18.0±2	51.4
CAG1-51-4 20 µg/ml	4±3	89.1
10 µg/ml	12.7±4	65.7
5 µg/ml	15.3±1.2	58.6
α-hCG 20 µg/ml	35.0±5	---
10 µg/ml	33.7±7	---
5 µg/ml	32.0±5	---

\* No. of syncytia from 5 randomly selected micro-  
scopical field at a magnification of 100X.

\*\* Results expressed in  $x \pm S.D.$ , n=3

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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CLAIMS

1. A monoclonal antibody which neutralizes HIV-1.
2. A monoclonal antibody of Claim 1 which neutralizes different strains and different isolates of HIV-1.
3. A monoclonal antibody of Claim 2 which is of mouse origin.
4. A monoclonal antibody of Claim 2 which is of IgG isotype.
5. A monoclonal antibody of Claim 2 which is a chimeric murine/human antibody.
6. A antigen binding fragment of the monoclonal antibody of Claim 2.
7. A monoclonal antibody which binds the gp 120 envelope protein of HIV-1 and inhibits the infection of human T cells by HIV-1.
8. A monoclonal antibody of Claim 7 which additionally inhibits infection of human T cells by fusion with HIV-1-infected cells.
9. A monoclonal antibody of Claim 8 which inhibits, with an  $IC_{50}$  of less than 10 ng/ml, the

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- infection of susceptible human T cells lines by HIV-1<sub>B</sub> at 20 times TCID<sub>50</sub> in a nine day assay.
10. A monoclonal antibody of Claim 8 which is of mouse origin.
  11. A monoclonal antibody of Claim 8 which is of IgG isotype.
  12. A monoclonal antibody of Claim 8 which is a chimeric murine/human antibody.
  13. A monoclonal antibody of Claim 8 which is produced by a hybridoma which is formed from the fusion of a murine lymphocyte and a murine myeloma.
  14. The monoclonal antibodies antibodies BAT085, BAT123, BAT267, and BAT509.
  15. A bispecific antibody comprising two different antigen binding portions, one derived from a monoclonal anti-HIV-1 antibody which binds the gp 120 envelope protein of HIV-1 and inhibits the infection of T cells by HIV-1 and the second being of different specificity.
  16. A bispecific antibody of Claim 15 wherein the monoclonal anti-HIV-1 antibody additionally inhibits infection of susceptible human T cell lines by fusion with HIV-1-infected cells.



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17. A bispecific antibody of Claim 15, wherein the anti- HIV-1 antibody is selected from the group consisting of BAT123, BAT267 and BAT509.
18. A bispecific antibody of Claim 15 wherein the second antigen-binding portion is specific for a surface antigen of a human T cell or macrophage.
19. A bispecific antibody of Claim 18 wherein the surface antigen is the CD4 molecule.
20. An antibody conjugate comprising a monoclonal antibody which neutralizes HIV-1 conjugated with a cytotoxic agent, an anti-viral agent or an agent which facilitates passage through the blood-brain barrier.
21. An antibody conjugate of Claim 20 wherein the antibody neutralizes different strains and different isolates of HIV-1.
22. An antibody conjugate of Claim 20 wherein the monoclonal antibody is selected from the group consisting of BAT085, BAT123, BAT267 and BAT509.
23. An antibody conjugate of Claim 20 wherein the cytotoxic or anti-viral agent is selected from the group consisting of cytotoxic steroids, galanin, abrin, ricin, phospholipases,

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interferons, radioactive nuclides, azido-thymidine, or ribovirin.

24. A microcarrier impregnated with a monoclonal antibody which neutralizes HIV-1.
25. A microcarrier of Claim 24 wherein the monoclonal antibody neutralizes different strains and different isolates of HIV-1.
26. A microcarrier of Claim 24 wherein the monoclonal antibody is selected from the group consisting of BAT085, BAT123, BAT267 and BAT509.
27. A microcarrier of Claim 24 which is a liposome.
28. A microcarrier having conjugated to its surface a monoclonal antibody which neutralizes HIV-1 and having a cytotoxic or anti-viral agent associated therewith.
29. A microcarrier of Claim 28 wherein the monoclonal antibody neutralizes different strains and different isolates of HIV-1.
30. A microcarrier of Claim 28 wherein the monoclonal antibody is selected from the group consisting of BAT085, BAT123, BAT267, and BAT509.

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31. A microcarrier of Claim 28 wherein the agent is one or more of cytotoxic steroids, galanin, abrin, ricin, phospholipases, interferons, radioactive nuclides, azidothymidine, deoxycytidine or ribovirin.
32. A method of treating an HIV-1 infected individual comprising administering to the individual a therapeutic amount of a monoclonal antibody which neutralizes HIV-1.
33. A method of Claim 32 wherein the individual is an HIV-1 carrier or a patient with AIDS or AIDS-related complex.
34. A method of Claim 32 wherein the monoclonal antibody neutralizes different strains and different isolates of HIV-1.
35. A method of Claim 32 wherein the monoclonal antibodies is selected from the group consisting of BAT085, BAT123, BAT267, and BAT509.
36. A method of treating an individual who is an HIV-1 carrier or is a patient with AIDS or AIDS-related complex, comprising administering to the individual a therapeutic amount of a monoclonal anti-HIV-1 antibody which binds the gp120 envelope protein of HIV-1, inhibits the infection of T cells by HIV-1, and inhibits

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infection of T cells by fusion with HIV-1-infected cells.

37. A method of treating an HIV-1 infected individual, comprising administering a therapeutic amount of a bispecific antibody comprising two different antigen binding portions, one derived from a monoclonal anti-HIV-1 antibody which binds the gp 120 envelope protein of HIV-1 and inhibits the infection of T cells by HIV-1 and the second being of different specificity.
38. A method of Claim 37, wherein the anti-HIV-1 antibody is selected from the group consisting of BAT085, BAT123, BAT267 and BAT509.
39. A method of Claim 38, wherein the second antigen-binding portion is specific for a surface antigen a human T cell or macrophage.
40. A method of Claim 39, wherein the surface antigen is the CD4 molecule.
41. A method of treating an HIV-infected individual comprising administering to the individual a therapeutic amount of an antibody conjugate comprising a monoclonal antibody which neutralizes HIV-1 conjugated with a cytotoxic agent, an anti-vital agent or an agent which

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facilitates passage through the blood-brain barrier.

42. A method of Claim 42, wherein the antibody neutralizes different strains and different isolates of HIV-1.
43. A method of Claim 42, wherein the monoclonal antibody is selected from the group consisting of BAT085, BAT123, BAT267 and BAT509.
44. A method of Claim 41, wherein the cytotoxic or anti-viral agent is selected from the group consisting of cytotoxic steroids, galanin, abrin, ricin, phospholipases, interferons, radioactive nuclides, azidothymidine, deoxycytidine or ribovirin.
45. A method of passively immunizing an individual against HIV-1 comprising administering to the individual a monoclonal antibody which neutralizes HIV-1.
46. A method of Claim 45 wherein the monoclonal antibody neutralizes different strains and different isolates of HIV-1.
47. A method of Claim 45 wherein the monoclonal antibodies is selected from the group consisting of BAT123, BAT267, and BAT509.

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48. A method of Claim 45 wherein the individual is also administered an antigen which induces active immunization against HIV-1.
49. A method of Claim 45 wherein the individual is HIV-1 infected but asymptomatic.
50. An anti-idiotypic antibody specific for an idiotype of a monoclonal antibody which neutralizes different strains and different isolates of HIV.
51. An anti-idiotypic antibody of Claim 50, which is a chimeric animal/human antibody.
52. A anti-idiotypic antibody of Claim 51, which is a chimeric murine/human antibody.
53. An anti-idiotypic antibody specific for the idiotype of an antibody selected from the group consisting of BAT085, BAT123, BAT267 and BAT509.
54. A method of immunizing against HIV-1 comprising administering an immunogenic amount of an anti-idiotypic antibody specific for an idiotype of a monoclonal antibody which neutralizes different strains and different isolates of HIV.

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55. A continuous, stable antibody-producing cell line which produces a monoclonal antibody which neutralizes HIV-1.
56. A cell line of Claim 55, which is a hybridoma.
57. A cell line of Claim 55, which is a transfected myeloma which produces a chimeric animal/human antibody.
58. A cell line of Claim 57, wherein the chimeric antibody is a murine/human antibody.
59. A hybridoma cell line which produces an antibody that neutralizes different strains and different isolates of HIV-1.
60. A hybridoma cell line of Claim 59, which produces an antibody selected from the group consisting of BAT085, BAT123, BAT267 and BAT509.
61. A method of extracorporeal treatment of an HIV-infected individual comprising:
  - a) removing blood leukocytes from the individual;
  - b) contacting the removed leukocytes with a monoclonal antibody which neutralizes HIV-1; and
  - c) returning the leukocytes to the individual.

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62. A method of Claim 61, further comprising stimulating the leukocytes with an immunopotentiating drug.
63. A method of Claim 62, wherein the immunopotentiating drug is interleukin-2.
64. A method of Claim 62, wherein the monoclonal antibody is a murine/human chimeric antibody.
65. A method of Claim 62, wherein the monoclonal antibody neutralizes different strains and different isolates of HIV-1.
66. A method of Claim 62, wherein the monoclonal antibody is selected from the group consisting of BAT085, BAT123, BAT267 and BAT509.
67. An immunogenic peptide having an amino acid sequence which is the same or similar to an epitope bound by an anti-HIV antibody which neutralizes different strains and different isolates of HIV-1.
68. An immunogenic peptide of Claim 67 coupled with a carrier protein.
69. An immunogenic peptide of Claim 67 having the amino acid sequence RPNNTNRKSIRIQRGPGRAFVTIGK or any actual epitope portion thereof.



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70. An immunogenic peptide of Claim 67 coupled with a carrier protein.
71. A method of immunizing against HIV-1, comprising administering an immunogen composition comprising an immunogenic peptide having an amino acid sequence which is the same or similar to an epitope bound by an anti-HIV antibody which neutralizes different strains and different isolates of HIV-1 in a pharmaceutically acceptable vehicle
72. A method of Claim 71, wherein the peptide has the amino acid sequence RPNNNTRKSIRIQRGPGRAFTIGK.
73. A method of Claim 72, wherein the peptide is coupled to a carrier protein.
74. A method of Claim 71, wherein the peptide has the amino acid sequence RPNNNTRKSIRIQRG.
75. A method of Claim 71, wherein the peptide has the amino acid sequence SIRIQRGPGRAFTIGK.
76. A method of Claim 71, wherein the peptide has the amino acid sequence
77. A chimeric, viral-neutralizing immunoglobulin comprising a viral-specific antigen-binding



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region of non-human origin and a constant region of human origin.

78. A chimeric immunoglobulin of Claim 77, wherein the virus human immunodeficiency virus, human T cell lymphotropic virus I or hepatitis B virus.
79. A chimeric immunoglobulin of Claim 78, wherein the virus is human immunodeficiency virus.
80. A chimeric immunoglobulin of Claim 77, wherein the antigen-binding region is of murine origin.
81. A chimeric, HIV-neutralizing immunoglobulin comprising an HIV-specific antigen binding region of nonhuman origin and a human constant region.
82. A chimeric HIV-neutralizing immunoglobulin of Claim 5, which neutralizes different strains and different isolates of HIV-1.
83. A chimeric HIV-neutralizing immunoglobulin of Claim 82, which binds the gp120 envelope protein of HIV, inhibits the infection of T cells by HIV and inhibits the infection of T cells by fusion with HIV-infected cells.
84. A chimeric HIV-neutralizing immunoglobulin comprising:

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- a. at least one chimeric heavy chain comprising an antigen binding region derived from the heavy chain of a nonhuman HIV-neutralizing immunoglobulin linked to at least a portion of a human heavy chain constant region, the heavy chain being in association with;
  - b. at least one chimeric light chain comprising an antigen binding region derived from a light chain of the nonhuman immunoglobulin linked to at least a portion of a human light chain constant region.
85. A chimeric HIV-neutralizing immunoglobulin of Claim 84, which neutralizes different strains and different isolates of HIV-1.
86. A chimeric HIV-neutralizing immunoglobulin of Claim 84, wherein the antigen binding region is of murine origin.
87. A chimeric HIV-neutralizing immunoglobulin of Claim 84, which binds the gp120 envelope protein of HIV, inhibits the infection of T cells by HIV and inhibits the infection of T cells by fusion with HIV-infected cells.
88. A chimeric immunoglobulin comprising an antigen binding region derived from the murine HIV-neutralizing immunoglobulin BAT123 or an

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immunoglobulin reactive with the same epitope as BAT123 and a human constant region.

89. The chimeric immunoglobulin CA123-G1-26.
90. A fused gene encoding a chimeric light or heavy chain immunoglobulin comprising:
  - a. a first DNA sequence encoding an immunoglobulin variable region of an viral-neutralizing antibody of nonhuman origin linked to;
  - b. a second DNA sequence encoding a constant region of an immunoglobulin of human origin.
91. A fused gene of Claim 90, wherein the first DNA sequence encodes an immunoglobulin variable region of an HIV-neutralizing antibody.
92. A fused gene of Claim 91, wherein the HIV-neutralizing immunoglobulin neutralizes different strains and isolates of HIV-1.
93. A fused gene of Claim 92, wherein the HIV-neutralizing immunoglobulin is the murine immunoglobulin BAT123.
94. An expression vector containing the fused gene of Claim 90, in expressible form.

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95. An expression vector containing the fused gene of Claim 91, in expressible form.
96. A method of therapy or prophylaxis of viral infection, comprising administering an individual an anti-viral amount of a chimeric viral-neutralizing immunoglobulin, comprising a viral-specific antigen-binding region of non-human origin and a constant region of human origin.
97. A method of Claim 96, wherein the virus is human immunodeficiency virus, human T cell lymphotropic virus I or hepatitis B virus.
98. A method of Claim 96, wherein the virus is human immunodeficiency virus (HIV).
99. A method of treating an individual who is an HIV carrier or is a patient with AIDS or AIDS-related complex, comprising administering to the individual a therapeutic amount of a chimeric HIV-neutralizing immunoglobulin, the immunoglobulin comprising a viral-specific antigen-binding region of non-human origin and a constant region of human origin.
100. A method of Claim 99, wherein the chimeric HIV-neutralizing immunoglobulin neutralizes different strains and different isolates of HIV-1.

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101. A method of Claim 99, wherein the chimeric HIV-neutralizing immunoglobulin binds the gp120 envelope protein of HIV, inhibits the infection of T cells by HIV and inhibits the infection of T cells by fusion with HIV-infected cells.
102. A method of Claim 99 wherein the chimeric HIV-neutralizing immunoglobulin comprising an antigen binding region derived from the murine HIV-neutralizing antibody BAT123 and a human constant region.

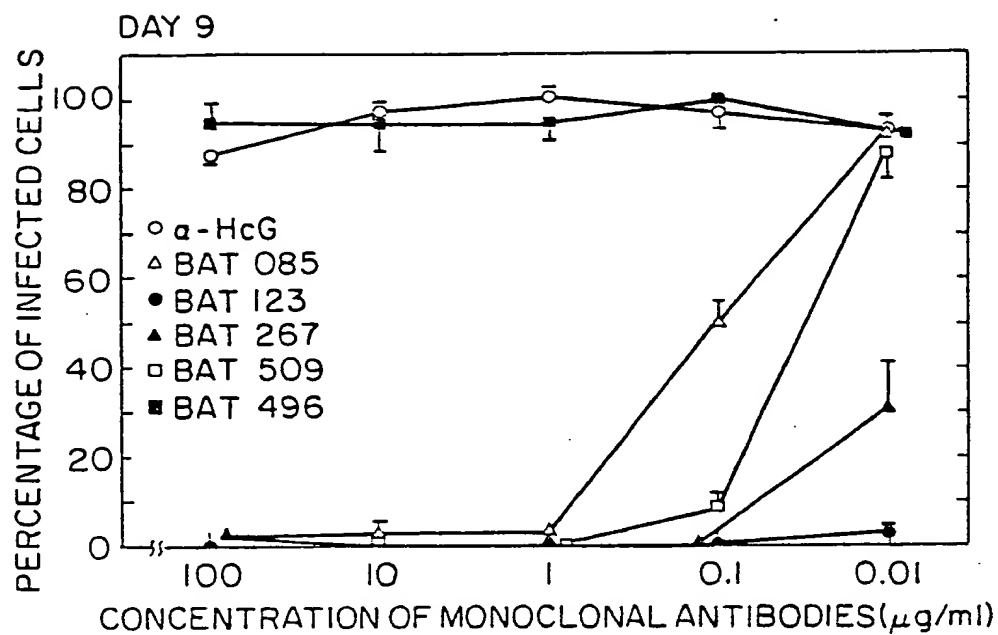


FIG. 1

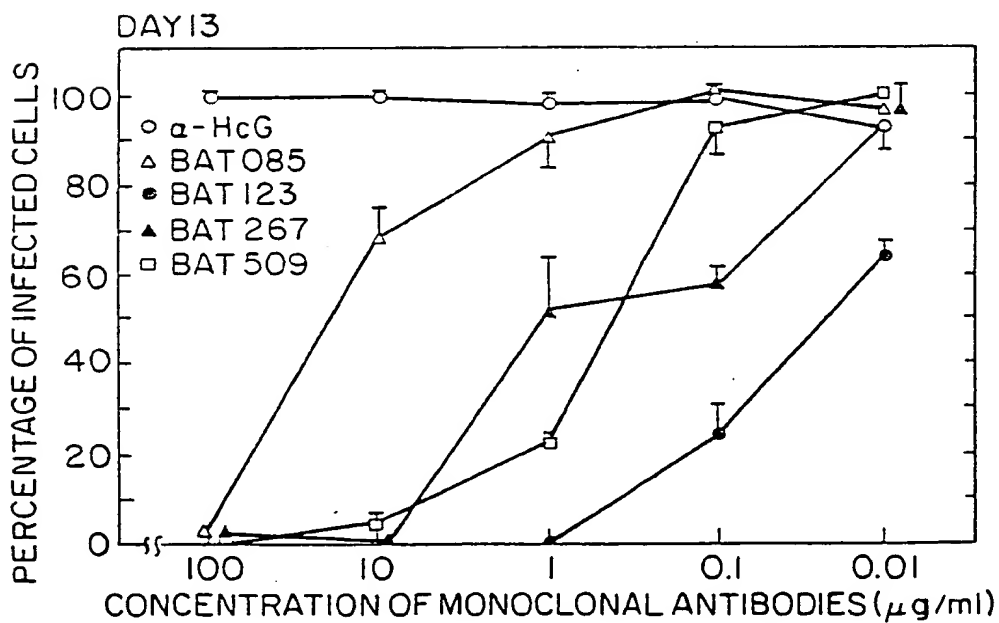
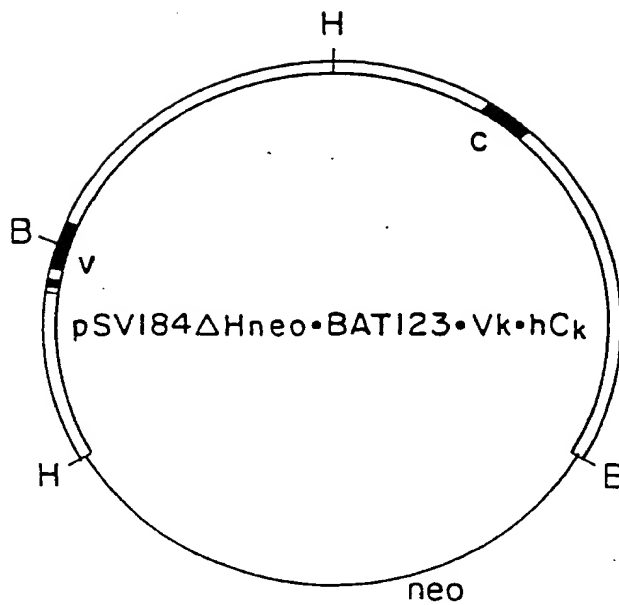


FIG. 2

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A



B

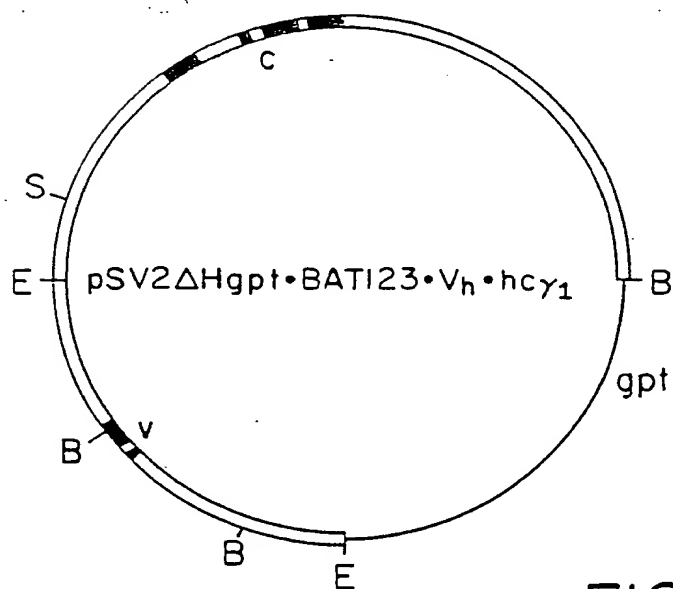


FIG. 3





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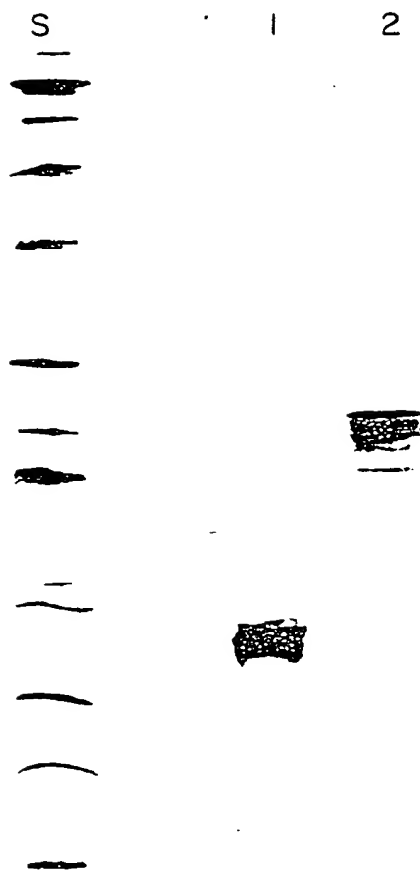


FIG. 4

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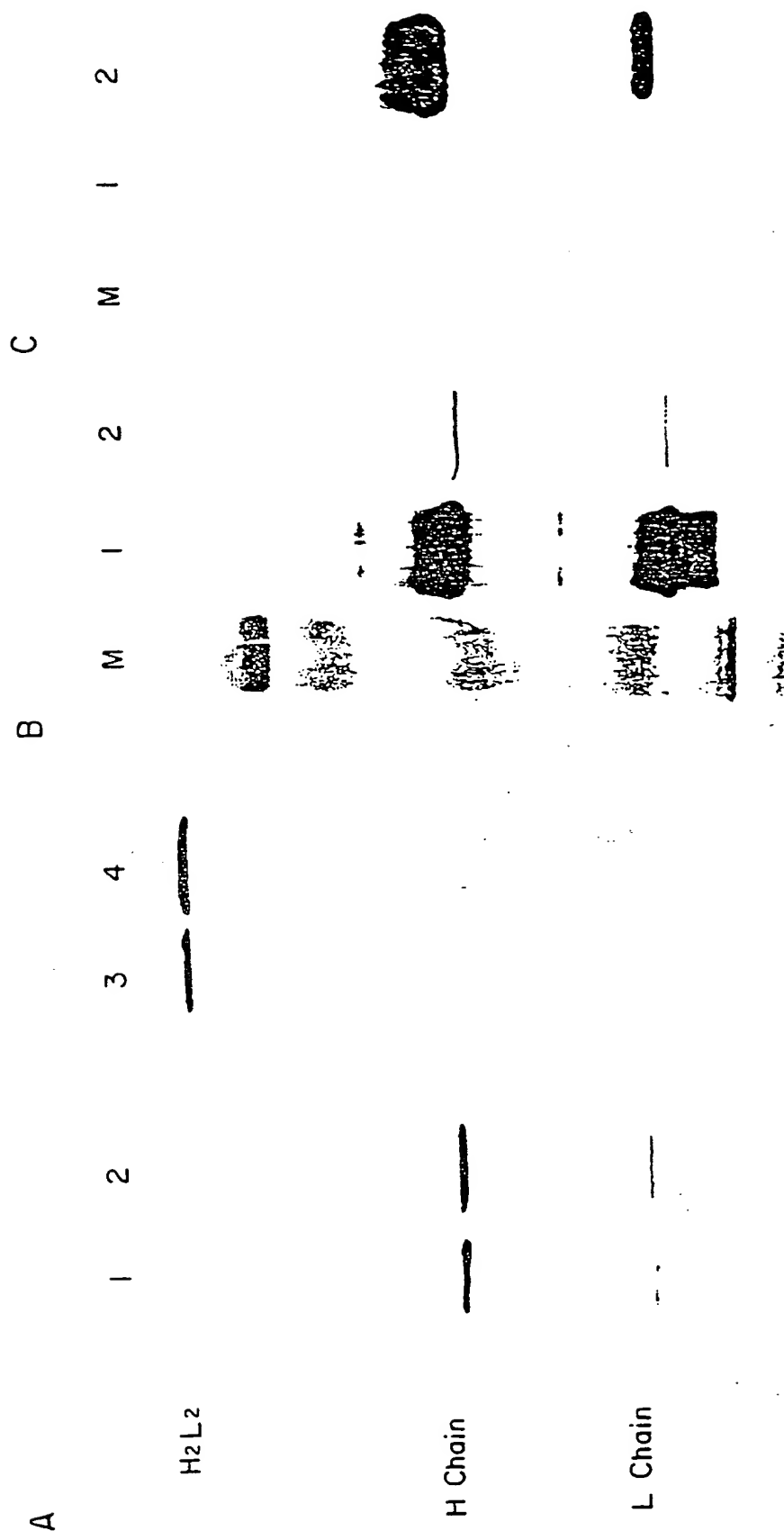


FIG. 5

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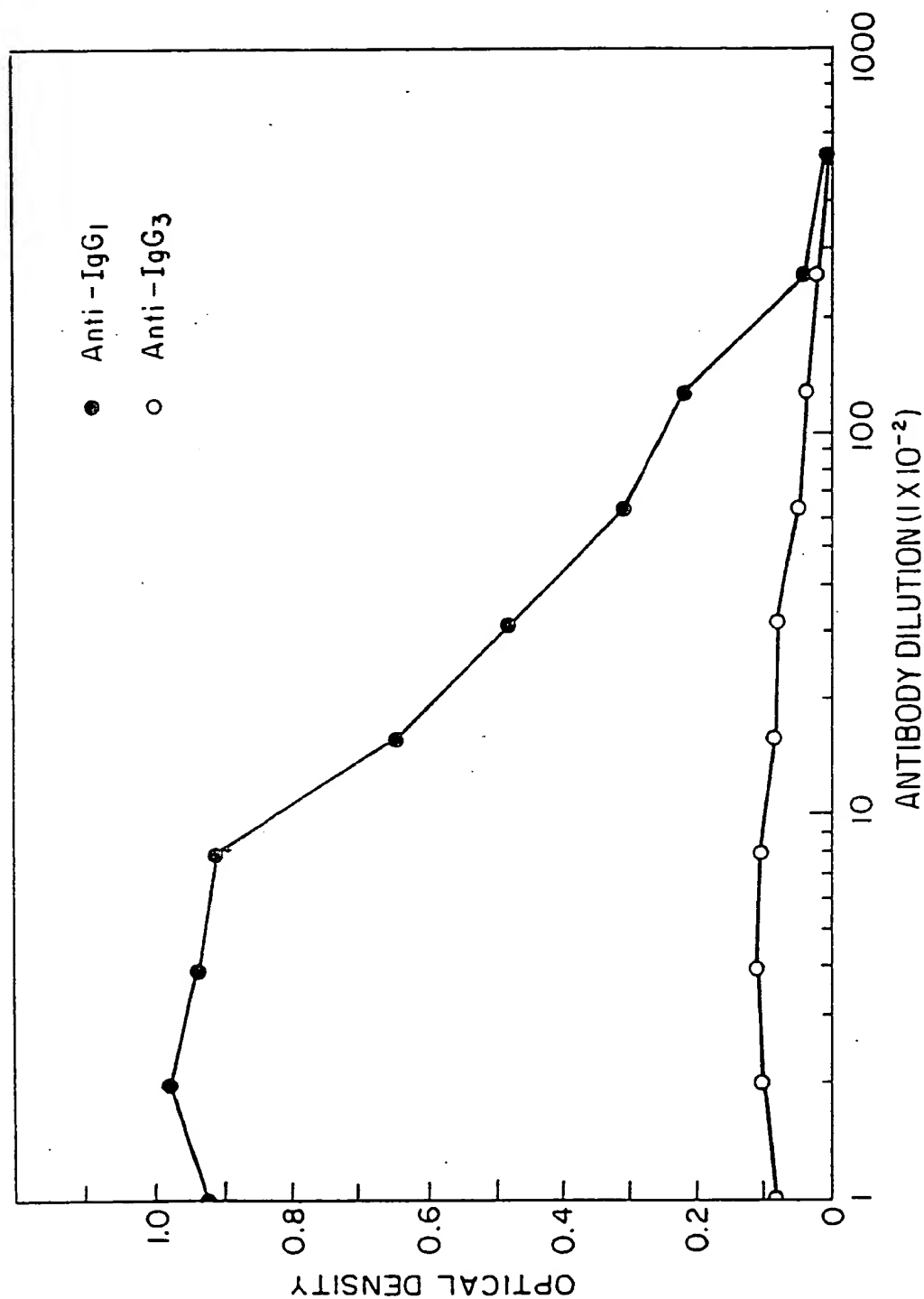


FIG. 6



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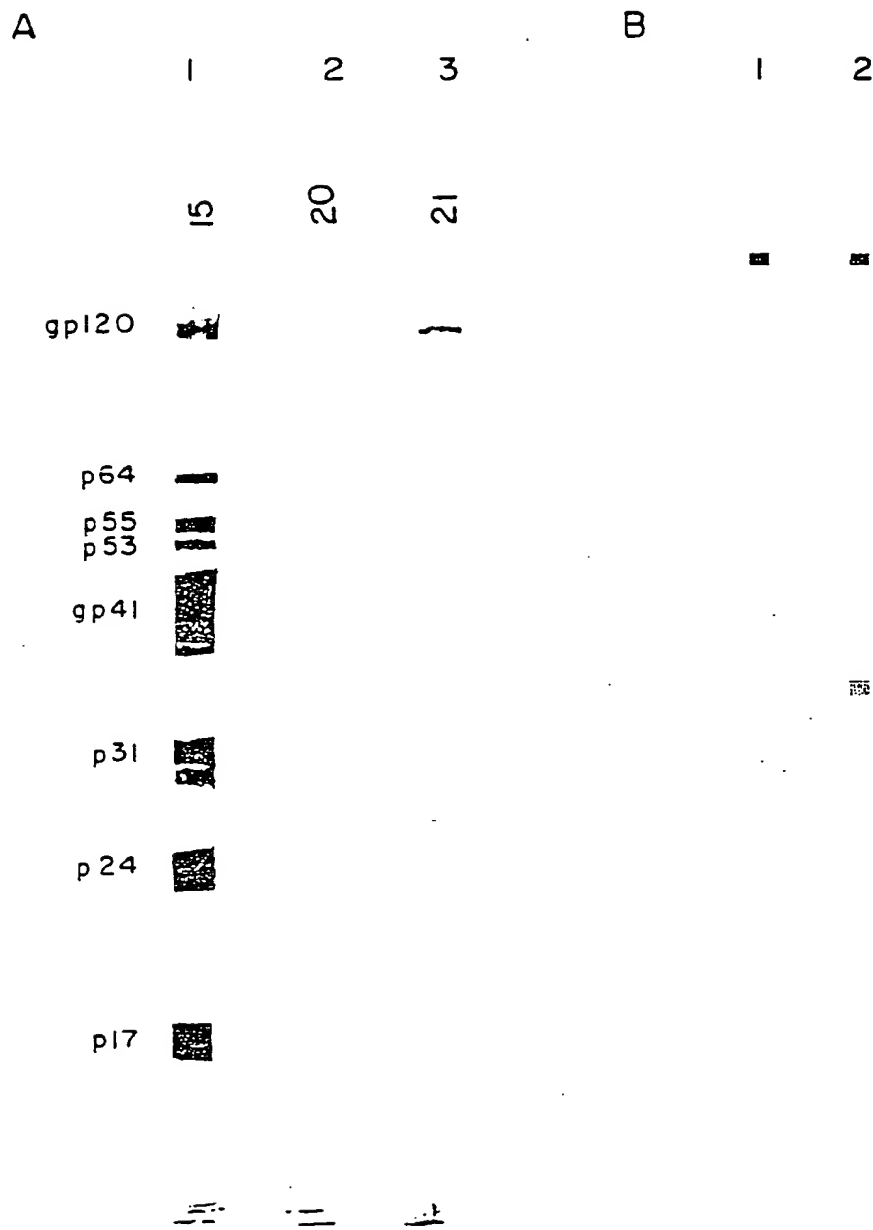


FIG.7

SUBSTITUTE SHEET

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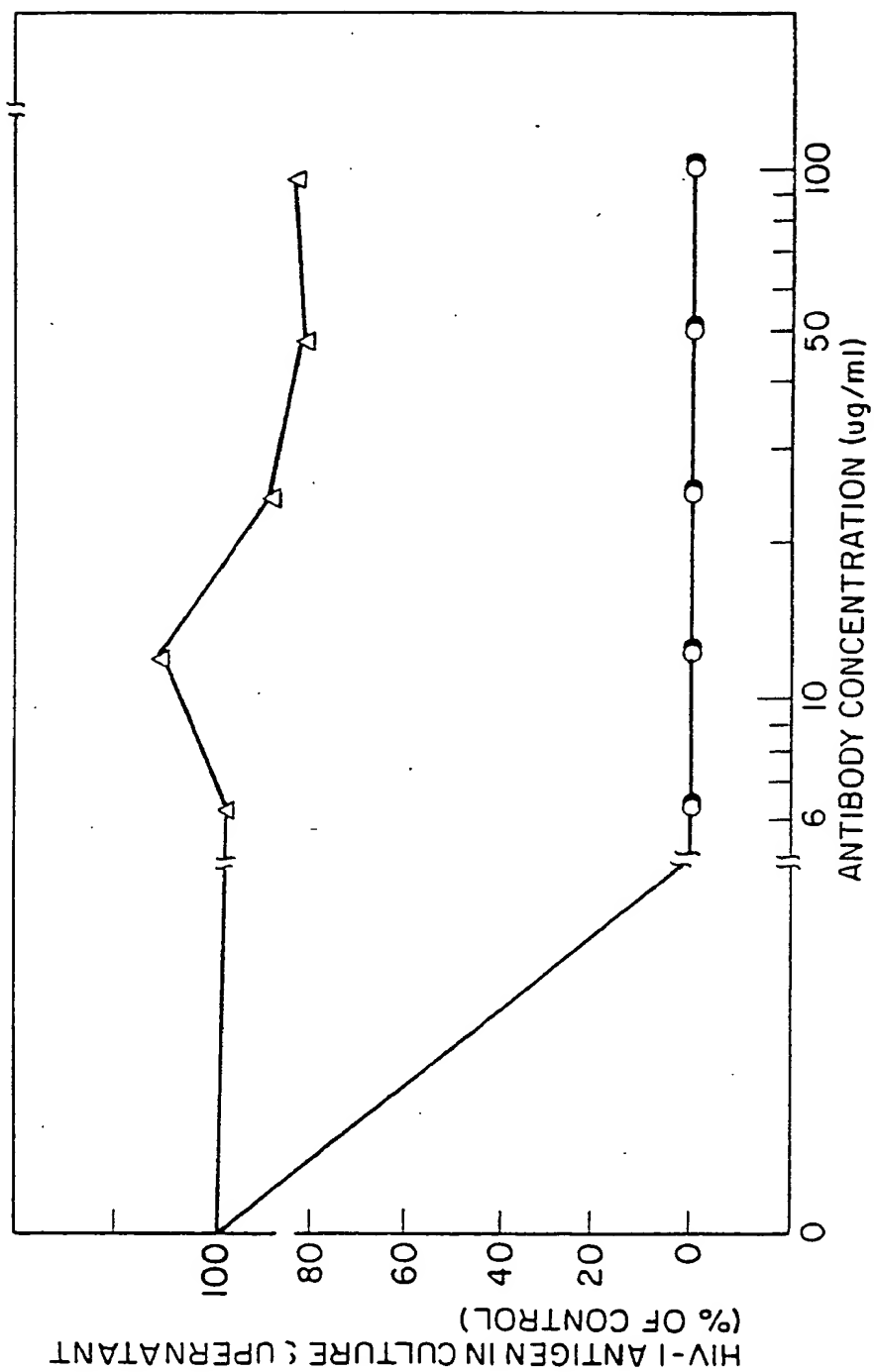


FIG. 8



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>4</sup> :</b> A61K 39/395, 39/42; C12N 15/00; C12P 21/00; A61K 39/21; C07K 7/08 C07K 7/10//G01N 33/569	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 88/ 09181</b>  <b>(43) International Publication Date:</b> 1 December 1988 (01.12.88)
<b>(21) International Application Number:</b> PCT/US88/01797 <b>(22) International Filing Date:</b> 27 May 1988 (27.05.88) <b>(31) Priority Application Numbers:</b> 057,445 137,861 197,766 <b>(32) Priority Dates:</b> 29 May 1987 (29.05.87) 24 December 1987 (24.12.87) 23 May 1988 (23.05.88) <b>(33) Priority Country:</b> US <b>(71) Applicants:</b> TANOX BIOSYSTEMS, INC. [US/US]; 10301 Stella Link Road, Houston, TX 77025 (US). BAYLOR COLLEGE OF MEDICINE [US/US]; 1200 Moursund Avenue, Houston, TX 77030 (US). <b>(72) Inventors:</b> CHANG, Tse, Wen.; 3323. Robinhood, Houston, TX 77005 (US). FUNG, Sek, C.; 3511 Deal, Houston, TX 77025 (US). SUN, Cecily, Rou-Yun; SUN, Bill, Nai-Chau; 3103 Nottingham Boulevard,		Houston, TX 77005 (US). CHANG, Nancy, T.; 3323 Robinhood, Houston, TX 77005 (US). LIOU, Ruey, Shyan; 1814 Riverbend Crossing, Sugarland, TX 77478 (US). ROSEN, Edward, M.; 2207 South Braeswood Boulevard, Houston, TX 77030 (US).  <b>(74) Agents:</b> DeCONTI, Giulio, A., Jr. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).  <b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 9 February 1989 (09.02.89)
<b>(54) Title:</b> MONOCLONAL ANTIBODIES NEUTRALIZING HIV-1  <b>(57) Abstract</b>  Monoclonal antibodies are disclosed which bind to the gp 120 protein on the envelope of HIV-1. These antibodies neutralize HIV-1. They inhibit the rate of infection of T cells, and also inhibit syncytium formation. Further, the antibodies are group-specific and neutralize different strains and isolates of HIV-1. These antibodies have a variety of uses, including the treatment and prevention of AIDS and ARC. Chimeric viral-neutralizing antibodies, including chimeric HIV-neutralizing antibodies, are also disclosed.		

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# INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/01797

**I. CLASSIFICATION F SUBJECT MATTER** (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: A 61 K 39/395; A 61 K 39/42; C 12 N 15/00; C 12 P 21/00; A 61 K 39/21;  
C 07 K 7/08; C 07 K 7/10/G 01 N 33/569

**II. FIELDS SEARCHED**

Minimum Documentation Searched \*

Classification System	Classification Symbols
IPC <sup>4</sup>	A 61 K; C 12 N; C 07 K; G 01 N

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

**III. DOCUMENTS CONSIDERED TO BE RELEVANT \***

Category *	Citation of Document, ** with Indication, where appropriate, of the relevant passages †	Relevant to Claim No. ‡
X	Eur. J. Immunol. 1986, Vol. 16, pages 1465-1468, T C Chanh et al.: "Human immunodeficiency virus gp 120 glycoprotein detected by a monoclonal antibody to a synthetic peptide", see page 1468, left coloumn	1-4,6-11,13, 55-59,67,68,70
X	EP, A, 0 214 709 (UNITED BIOMEDICAL INC.) 18 March 1987 whole document, see in particular column 26, lines 32-52	1-4,6-11,13, 55-59,67,68,70
X	The Journal of Biological Chemistry, Vol. 262, No. 12, 25 April 1987, pages 5769-5774, R C Kennedy et al.: "Use of a Resin-bound Synthetic Peptide for Identifying a Neutralizing Antigenic Determinant Associated with the Human Immunodeficiency Virus Envelope" see in particular page 5772, left column, figure 1 and tables I and II	1-4, 6-11,13, 55-59,67,68,70

\* Special categories of cited documents: †

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Δ" document member of the same patent family

**IV. CERTIFICATION**

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th November 1988	03 JAN 1989
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN



## III. D. CUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Science, Vol. 234, 12 December 1986, pages 1392-95 S D Putney et al.: "HTLV-III/LAV-Neutralizing Antibodies to an E.coli-Produced Fragment of the Virus Envelope" whole document	1-4,6-11,13, 55-59,67,68,70
Y	Nature, Vol. 324, 11 December 1986, pages 572-75 R A Weiss et al.: "Variable and conserved neutralization antigens of human immunodeficiency virus" whole document	1-4,6-11,13, 55-59,67,68,70
Y	Chemical Abstracts, Vol. 107, No. 3, 20 July 1987, (Columbus, Ohio, US), Ho David D et al.: "Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins", see page 460, abstract 21646h, & J. Virol. 1987, 61(6), pages 2024-8	1-4,6-11,13,14, 55-59,67-70
X	Proc. Natl. Acad. Sci. USA, Vol. 84, May 1987, pages 2951-55, P H Naylor et al.: "Human immunodeficiency virus contains an epitope immunoreactive with thymosin $\alpha_1$ and the 30-amino acid synthetic p17 group-specific antigen peptide HGP-30", see page 2951 and 2954	1,2,67
X	WO, A, 87/02775 (SOUTHWEST FOUNDATION FOR BIO-MEDICAL RESEARCH) 7 May 1987 whole document, see in particular page 27a, peptide 2, claim 11 page 5-7 and page 8, second paragraph	14,67-70
X	WO, A, 86/02383 (INSTITUT PASTEUR) 24 April 1986 see claims 8 and 9	67-70
P,X	DE, A, 3 727 703 (GENETIC SYSTEMS CORP.) 5 May 1988 whole document see in particular pages 22-24 and claims 9, 15-17	1-4,6-11,13,14, 55-60,66-70

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

PCT/US88/01  
SA 23557

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/10/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0214709	18-03-87	JP-A-62061996 US-A- 4735896	18-03-87 05-04-88
WO-A- 8702775	07-05-87	EP-A- 0245362 JP-T-63501716	19-11-87 14-07-88
WO-A- 8602383	24-04-86	FR-A- 2571968 EP-A- 0201540 JP-T-62500592 WO-A- 8604336 EP-A- 0211022 JP-T-62502095	25-04-86 20-11-86 12-03-87 31-07-86 25-02-87 20-08-87
DE-A- 3727703	05-05-88	FR-A- 2603107 LU-A- 86972 SE-A- 8703225 GB-A- 2196634 NL-A- 8701950	26-02-88 02-03-88 21-02-88 05-05-88 16-03-88

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers \* ) because they relate to subject matter not required to be searched by this Authority, namely:

\* ) 32-49, 61-66, 71-77 and 96-102

See PCT Rule 39.1 (iv)

Method for treatment of the human or animal body by means of surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

- claims 1-4, 6-11, 13, 14, 55-60 and 67-70: Monoclonal antibodies, cellines producing said antibodies and peptide antigens specific for said antibodies.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-4, 6-11, 13, 14, 55-60, 67-70.

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM THE supplemental sheet (2)

- claims 5, 12 and 77-83: Chimeric viral neutralizing immunoglobulin with nonhuman variable region and human constant region and fused gene therefore.
- claims 15-19 and 84-87: Bispecific monoclonal antibodies with two different antigen binding (variable) regions, chimeric immunoglobulins with one HIV specific heavy chain variable region (nonhuman origin) and one light chain with one nonhuman variable region (no specificity stated).
- claims 20-23 and 28-31: Monoclonal HIV neutralizing antibodies conjugated to cytotoxic agents, antiviral agents etc. and optionally to microcarrier also.
- claims 24-27: Microcarrier bound monoclonal HIV neutralizing antibodies.
- claims 50-53: Anti-idiotypic antibody with specificity for an idiotyp of a HIV neutralizing monoclonal antibody.